Université de Montréal

Caractérisation Biochimique et Physiologique de la Fonction Catalytique de l'Hexokinase dans la Racine de Pomme de Terre (*Solanum tuberosum*)

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Thèse présentée à la Faculté des Études Supérieures

en vue de l'obtention du grade de Philosophiæ Doctor (Ph.D.)

en Sciences Biologiques

août 2007

Université de Montréal

Faculté des Études Supérieures

Cette thèse intitulée :

Caractérisation Biochimique et Physiologique de la Fonction Catalytique de l'Hexokinase dans la Racine de Pomme de Terre (*Solanum tuberosum*)

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Résumé

La glycolyse végétale joue un rôle primordial dans l'apport d'énergie à la cellule et dans la production de squelettes carbonés pour de multiples voies de biosynthèses. Malgré son importance, les composantes de sa régulation *in vivo* et du contrôle de son flux demeurent largement inconnues. Cette problématique a inspiré la présente thèse, qui détermine la nature et le degré d'implication de l'hexokinase (HK) dans la régulation et le contrôle du flux de la glycolyse.

Article 1 – L'HK engage les hexoses dans la glycolyse en les phosphorylant sur leur sixième carbone. Outre cette fonction catalytique, l'HK remplit une fonction distincte dans la perception et la signalisation des hexoses. Cette enzyme existe sous plusieurs isoformes dans une variété d'organes et tissus végétaux. Toutefois, la signification physiologique d'une pluralité d'isoformes et leur contribution aux deux fonctions de l'HK restent mal comprises. Nous avons donc passé en revue la littérature suggérant que les profils d'expression, propriétés cinétiques et localisations subcellulaires des isoformes HK pourraient servir des rôles individuels et spécifiques. Notre revue souligne l'intérêt de poursuivre l'effort de caractérisation d'isoformes HK purifiées, et d'étudier conjointement toutes les isoformes d'un tissu afin d'estimer leurs parts dans les fonctions de l'HK. Nous avons également constaté le manque de données qualitatives et quantitatives sur l'implication de l'HK dans la régulation de la glycolyse et le contrôle de son flux. *Article 2* – Compte tenu des conclusions de notre revue, nous avons caractérisé ScHK2, une HK de *Solanum chacoense* utilisée dans ce projet, afin d'en connaître les propriétés cinétiques. Après clonage et séquençage de son ADNc, nous avons exprimé et purifié une version recombinante de ScHK2 jusqu'à homogénéité électrophorétique, avec une activité spécifique de 5,3 U/mg protéine. Il s'agit de la première caractérisation cinétique d'une HK recombinante pure de plante. D'après ses propriétés cinétiques et sa séquence déduite, ScHK2 pourrait faciliter l'import d'hexoses dans les cellules puits en maintenant un gradient de concentration de part et d'autre de la membrane plasmique à laquelle elle serait associée. ScHK2 semble donc vouée à la fonction catalytique de l'HK en tant qu'enzyme glycolytique. *Article 3* – Nous avons tenté de mieux apprécier l'implication de l'HK dans la régulation de la glycolyse et le contrôle de son flux. Les niveaux d'activité HK de racines

de pomme de terre (*S. tuberosum*) ont été manipulés à cette fin, par transformation avec des constructions sens ou antisens de l'ADNc de ScHK2. Nous avons généré 23 clones racinaires aux niveaux d'activité HK compris entre 72% et 800% de ceux chez 3 contrôles transformés avec un vecteur vide. D'après des essais menés sur 18 enzymes du métabolisme primaire, seule l'HK variait significativement dans la population de clones. De plus, la hausse d'activité HK parmi les clones tendait à limiter leur croissance, démontrant ainsi un rôle majeur de l'HK dans la régulation de la croissance racinaire. Nous avons quantifié pour la première fois le contrôle de l'HK sur l'amont de la glycolyse végétale selon l'Analyse de Contrôle Métabolique, et avons mesuré un coefficient de contrôle de flux de 0,76 ± 0,08. Cette valeur particulièrement élevée traduit un fort contrôle de l'HK sur l'étape de glycolyse qu'elle catalyse. Toutefois, les niveaux d'hexoses-phosphates étaient similaires parmi les clones ce qui, d'après nos données de marquages radioactifs, serait dû à leur recyclage en hexoses et D-saccharose par des cycles de substrats (dits "futiles"). Nous avons conclu que le contrôle de l'HK sur le flux glycolytique se limite probablement à sa propre étape. Par contre, l'HK semble impliquée dans la régulation glycolytique en modulant le flux parcourant les cycles de substrats auxquels elle prend part. Le coût en ATP associé à ces flux cycliques expliquerait les variations de croissance et de niveaux de métabolites parmi les clones racinaires. La régulation glycolytique par les cycles de substrats serait une variante végétale parmi divers systèmes de régulation eucaryotes évitant une hausse excessive du flux qui bloquerait la voie par séquestration du phosphate à l'étape de l'HK.

Sur la base des trois articles présentés, nous discutons des enjeux et défis de l'élucidation des modes d'action des isoformes HK en vue de mieux comprendre leurs contributions respectives aux fonctions de l'HK. Ces recherches incitent à étudier d'autres enzymes de la glycolyse selon la même approche afin de compléter le schéma global de sa régulation et du contrôle de son flux.

Mots-clés : Analyse de contrôle métabolique ; Cinétique enzymatique ; Coefficient de contrôle de flux ; Cycle de substrats (dit "futile") ; 2-Désoxy-D-glucose ; Glycolyse ; Hexokinase ; Isoforme ; Métabolisme primaire ; *Solanum tuberosum* ; Transgénèse.

Abstract

Plant glycolysis plays a primordial role in yielding energy to the cell and in producing carbon building blocks for multiple biosynthetic pathways. Despite its importance in plant metabolism, the components of its *in vivo* regulation and flux control remain largely unknown. These issues have inspired the present thesis, which determines the nature and degree of implication of hexokinase (HK) in glycolytic regulation and flux control.

Article 1 – HK phosphorylates hexoses on their sixth carbon and commits them to glycolysis. Beyond its catalytic function, HK fulfills a separate function in hexose sensing and signaling. This enzyme exists as several isoforms in a variety of plant organs and tissues. However, the physiological relevance of multiple HK isoforms and their contribution to either function remains poorly understood. Therefore, we have reviewed the literature to discuss how the expression patterns, kinetic properties and subcellular localizations of HK isoforms may serve individual, specific roles. Our review underscores the interest of pursuing the characterization of purified HK isoforms, and of studying all isoforms of a tissue at once to outline their parts in HK functions. Lastly, we have noted the lack of qualitative and quantitative data regarding the implication of HK in glycolytic regulation and flux control. *Article 2* – Taking in account the conclusions of our review, we have characterized ScHK2, an HK from *Solanum chacoense* used in this project, to determine its kinetic properties. After cloning and sequencing its cDNA, we have expressed and purified a recombinant version of ScHK2 to electrophoretic homogeneity, with a specific activity of 5.3 U/mg protein. This is the first kinetic characterization of a pure recombinant plant HK. Based on its kinetic properties and deduced sequence, ScHK2 may facilitate hexose import into sink cells by maintaining a concentration gradient across the plasma membrane, with which it may be associated. Therefore, ScHK2 appears to be dedicated to the catalytic function of HK as a glycolytic enzyme. *Article 3* – We have attempted to better understand the implication of HK in glycolytic regulation and flux control. HK activity levels of potato (*S. tuberosum*) roots were manipulated for this purpose by transformation with sense and antisense constructs of *ScHK2* cDNA. We have generated 23 root clones with HK activity levels ranging from 72% to 800% of those in 3 controls

transformed with an empty vector. According to assays carried out on 18 enzymes of primary metabolism, only HK activity varied significantly among the transformants. Furthermore, the rise in HK activity among the clones tended to limit their growth, thus demonstrating a major role for HK in root growth regulation. We have quantified for the first time the control of HK over the upstream portion of plant glycolysis according to Metabolic Control Analysis, and have measured a flux control coefficient of 0.76 ± 0.08. This particularly high value reflects a tight control of HK over the glycolytic step that it catalyzes. However, the hexose-phosphate levels were similar among the clones, which according to our radiolabeling data may be due to their recycling to hexoses and D-sucrose via substrate ('futile') cycles. We have concluded that the control of HK over glycolytic flux is probably restricted to its own step. By contrast, HK may be involved in glycolytic regulation by modulating the flux through the substrate cycles it is part of. The cost in ATP associated with these cycling fluxes may then explain the variations in growth and metabolite levels among the root clones. Glycolytic regulation by these substrate cycles may be a plant feature among various eukaryotic regulatory systems suited to avoid an excess in flux that would otherwise lead to a blockage of the pathway by phosphate sequestration at the HK step.

Based on the three articles presented, we discuss the advantages and challenges of elucidating the modes of action of HK isoforms in order to better understand their respective contributions to HK functions. This research suggests that a similar approach be taken with other glycolytic enzymes to complement the global scheme of glycolytic regulation and flux control.

Keywords: 2-Deoxy-D-glucose; Enzyme kinetics; Flux control coefficient; Glycolysis; Hexokinase; Isoform; Metabolic control analysis; Primary metabolism; *Solanum tuberosum*; Substrate (or 'futile') cycle; Transgenesis.

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Liste des abréviations

1,3BPGA (ou 1,3-DPGA)	1,3-bisphospho-D-glycérate
2-PGA	2-phospho-D-glycérate
3-PGA	3-phospho-D-glycérate
3-PGA kinase	3-phospho-D-glycérate kinase
3-PGA mutase	3-phospho-D-glycérate mutase
α-KG	α-cétoglutarate
ε-CA	acide ε-amino-*N*-caproïque
acétylCoA	acétyl-coenzyme A
AGPase	ADP-D-glucose pyrophosphorylase
ALD	aldolase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	albumine sérique bovine
*Cla*I	endonucléase I de *Caryophanon latum*
CAB2/3	protéine 2/3 de liaison à la chlorophylle *a/b*
DEAE	diéthylaminoéthyl
DOG	2-désoxy-D-glucose
DOG6P	2-désoxy-D-glucose-6-phosphate
DHAP	dihydroxyacétone-phosphate
DTT	dithiothréitol
*Eco*RI	endonucléase I d'*Escherichia coli* RY13
*Eco*RV	endonucléase V d'*Escherichia coli* B946
EDTA	acide éthylènediaminotétraacétique (sel disodique)
EGTA	acide éthylèneglycol-bis-(aminoéthyléther)-tétraacétique
*Ehe*I	endonucléase I d'*Erwinia herbicola* 9/5
ER	réticulum endoplasmique
EST	marqueur de séquence exprimée
F1P	D-fructose-1-phosphate
F1,6BP (ou Fru-1,6-P_2)	D-fructose-1,6-bisphosphate
F6P	D-fructose-6-phosphate

FBPase	D-fructose-1,6-bisphosphatase
FCC	coefficient de contrôle de flux
FK	fructokinase
FPLC	chromatographie rapide de protéines en phase liquide
Fru	D-fructose
G1P	D-glucose-1-phosphate
G3P	D-glycéraldéhyde-3-phosphate
G6P	D-glucose-6-phosphate
G6PDH	D-glucose-6-phosphate déshydrogénase
GABA	acide γ-aminobutyrique
GFP	protéine fluorescente verte
GK	glucokinase
GKRP	protéine régulatrice de la GK
Glc	D-glucose
Glc-P	D-glucose-1-phosphate ou D-glucose-6-phosphate
GMPase	GDP-D-mannose pyrophosphorylase
GOGAT	Glu synthase (Gln:2-oxoglutarate aminotransférase)
GPT	translocateur de D-glucose-6-phosphate/Pi
GS	Gln synthétase
Hepes	acide *N*-(2-hydroxyéthyl)-1-pipérazine-*N'*-2-éthanesulfonique
hexose-P	hexose-phosphate
*Hin*cII	endonucléase II d'*Haemophilus influenzae* Rc
*Hin*dIII	endonucléase III d'*Haemophilus influenzae* Rd
HK	hexokinase
*Hpa*I	endonucléase I d'*Haemophilus parainfluenzae*
HPLC	chromatographie en phase liquide à haute pression
IgG	immunoglobuline G
INV	invertase
IPTG	isopropyl β-D-1-thiogalactopyranoside
K_{mapp}	K_m apparent
*Kpn*I	endonucléase I de *Klebsellia pneumoniae* OK 8

LB	Luria–Bertani
LDH	lactate déshydrogénase
MDH	malate déshydrogénase
MES	acide 2-(*N*-morpholino)-éthanesulfonique
MK	mannokinase
MS	Murashige et Skoog
MTD	mannitol déshydrogénase
MYA	mannitol/extrait de levure/sulfate d'ammonium
NADP-GAPDH (NP)	G3P déshydrogénase NADP-dépendante, non phosphorylante
NAD(P)-ME	enzyme malique NAD(P)-dépendante
NBT	nitrobleu de tétrazolium
NDPK	nucléoside-5'-diphosphate kinase
Ni–NTA	acide Ni^{2+}–nitrilotriacétique
npt-II	gène de la néomycine phosphotransférase II
NR	nitrate réductase
OAA	oxaloacétate
OPPP	voie d'oxydation des pentoses-phosphates
MCP	mort cellulaire programmée
PCR	réaction en chaîne de l'ADN polymérase
PEP	phospho*énol*pyruvate
PEPase	phospho*énol*pyruvate phosphatase
PEPC	phospho*énol*pyruvate carboxylase
PFK	phosphofructokinase ATP-dépendante
PFP	pyrophosphate:F6P 1-phosphotransférase
PGI	phosphoglucose isomérase
pGlcT	transporteur putatif de D-glucose
PGM	phosphoglucomutase
(P)P_i	(pyro)phosphate inorganique
PK	pyruvate kinase
PMI	phosphomannose isomérase
PMM	phosphomannomutase

PMSF	fluorure de phénylméthylsulfonyl
PPDK	pyruvate, phosphate dikinase
PPT	translocateur de phospho*énol*pyruvate/ phosphate inorganique
PTP	pore de transition de perméabilité
PVDF	difluorure de polyvinylidène
PVPP	polyvinylpolypyrrolidone insoluble
RFO	oligosaccharides de la famille du raffinose
RPT5B	sous-unité 5B de l'ATPase AAA de la particule régulatrice 19S du protéasome
SDS	dodécylsulfate de sodium
SDS–PAGE	électrophorèse sur gel de polyacrylamide–SDS
SE	erreur standard
SPS	D-saccharose-phosphate synthase
SSC	citrate de sodium salin
SST	D-saccharose–D-saccharose fructosyltransférase
Suc	D-saccharose
SuSy	D-saccharose synthase
T6P	D-tréhalose-6-phosphate
TBST	Tween 20 salin tamponné au Tris
TCA (cycle)	cycle des acides tricarboxyliques (ou cycle de Krebs)
TGD	transglucosidase
TPI	triose-phosphate isomérase
TPT	translocateur de trioses-phosphates/ phosphate inorganique
triose-P	triose-phosphate
UDP-Glc (ou UDP-glucose)	UDP-D-glucose
UGPase	UDP-D-glucose pyrophosphorylase
U	unité
VDAC	canal à anions voltage-dépendant (appelé aussi porine)
VHA-B1	H^+-ATPase vacuolaire B1
*Xba*I	endonucléase I de *Xanthomonas badrii*
*Xho*I	endonucléase I de *Xanthomonas holcicola*

"Regulation is not a later development superposed on metabolism after catalysis had become well-established – a mere refinement added to an already functioning system. Regulation is the most fundamental difference between living and nonliving systems, and it must have coevolved with other properties of life from the beginning."

Atkinson, 1977

Remerciements

Je tiens tout d'abord à remercier mon directeur de thèse, le Dr. Jean Rivoal, de m'avoir supervisé durant mes études de Doctorat, et d'avoir pourvu les fonds nécessaires à mes travaux de recherche.

Je me dois de remercier les Comités de Bourse d'accueil, de Bourse J. Rousseau, de Bourse de rédaction et de Bourse de fin d'études du FICSUM, pour leur soutien financier. Merci, aussi, aux membres de mon Comité Conseil, les Drs. Mario Jolicœur et Deep Saini, pour leurs suggestions et leur disponibilité. Merci, encore, aux Drs. Mario Capadoccia, Mario Jolicœur et R. David Law pour le temps qu'ils auront consacré à évaluer cette thèse.

J'adresse tous mes remerciements aux membres du laboratoire J. Rivoal. À la Dre. Sonia Dorion, pour son aide experte, généreuse et constante à la paillasse. À Stéphanie Buisson pour le don de clones racinaires pour mon projet de recherche. À celles et ceux que j'ai eu le plaisir de côtoyer durant mes recherches : Evgenia Auslender, Rachel Beaubien, Guylaine Benoît, Audrey Clendenning, Mathieu Cloutier, Julie Jeukens, Geneviève Lévesque, Émilie Maheux, Marie-Claude Moisan, Adrián Troncoso Ponce et Hui Zhang.

Les personnes ressources de l'IRBV et du PMV ont facilité ma vie d'étudiant bien des fois, et je les en remercie. Je suis particulièrement reconnaissant aux Drs. Thérèse Cabana, François-Joseph Lapointe, Daniel Matton et Deep Saini pour leurs conseils et leur écoute. Merci, également, à Hélène Lavigne pour sa bonne humeur au travail, et à Claude Lafleur pour son aide lors de mon arrivée à Montréal.

J'adresse un grand merci à mes camarades étudiants et aux amis que je compte parmi eux : ils font de l'IRBV un lieu d'études exceptionnel.

Enfin et surtout, j'exprime ma plus profonde gratitude à ma famille et à mes amis, pour leurs encouragements et leur soutien à l'épreuve du temps ou de la distance qui nous sépare. J'adresse une pensée particulière à mon grand-père, Jean Rafron, à qui je dédie cette thèse.

Chapitre 1.

Introduction

1.1 L'importance de la glycolyse dans le métabolisme végétal découle de son organisation

1.1.1 La glycolyse végétale, noyau central d'un réseau métabolique

La glycolyse, ou voie de Embden–Meyerhof–Parnas, a été élucidée chez la levure de boulanger (*Saccharomyces cerevisiae*) et dans des tissus musculaires de mammifères vers la fin des années 1930 (Kresge *et al.*, 2005). Son existence dans les végétaux n'a cependant été démontrée qu'une quinzaine d'années plus tard (Givan, 1999). Aujourd'hui, il est clairement établi que la glycolyse occupe une place centrale dans le métabolisme de tous les êtres vivants, quasiment, même si elle n'est pas toujours présente dans son intégralité (Plaxton, 1996). Dans sa représentation classique, la glycolyse consiste en l'oxydation du D-glucose (Glc) en pyruvate, qui s'accompagne d'une réduction de NAD^+ en NADH et d'une production d'ATP :

$$1\ Glc + 2\ ADP + 2\ P_i + 2\ NAD^+ \rightarrow 2\ pyruvate + 2\ ATP + 2\ NADH, H^+ + 2\ H_2O \quad (1.1)$$

L'équation bilan (1.1) reflète l'implication de la glycolyse dans la production d'énergie (ATP) et de pouvoir réducteur (NADH) puisque le pyruvate est un substrat majeur de la respiration (Plaxton, 1996). Chez les végétaux, la glycolyse produit du pyruvate à partir du phospho*énol*pyruvate (PEP), mais aussi de l'oxaloacétate (OAA) et du malate qui sont transportés dans la mitochondrie et intégrés au cycle de Krebs (Givan, 1999). Quelque soit le flux les traversant, ces différentes branches de la glycolyse végétale fournissent généralement la totalité du carbone au cycle de Krebs. Le recours à d'autres voies cataboliques est plus rare, comme la β-oxydation des acides gras durant la germination et la sénescence (Salon *et al.*, 1988; Dieuaide-Noubhani *et al.*, 1995; Dieuaide-Noubhani *et al.*, 1997; Dennis et Blakeley, 2000). La glycolyse joue donc un rôle crucial dans l'apport de substrats à la respiration chez les plantes (Plaxton, 1996). Son autre rôle est anabolique : la glycolyse est une source de squelettes carbonés pour de multiples voies de biosynthèses (Tableau 1.1), ce qui la place au cœur d'un important réseau métabolique. Parmi les voies alimentées par la glycolyse, citons la synthèse de polysaccharides pariétaux, d'amidon, d'acides aminés, d'acides gras, la voie du shikimate ou encore la voie d'oxydation des pentoses-phosphates ("oxidative pentose-phosphate pathway", OPPP) (Tableau 1.1). La

glycolyse génère aussi du pouvoir réducteur nécessaire à ces biosynthèses, de manière directe (équation [1.1]) ou indirecte. En effet, le D-glucose-6-phosphate (G6P) glycolytique alimente l'OPPP, qui génère du NADPH pour la synthèse d'acides gras et l'assimilation du nitrite en Glu dans les plastes non-photosynthétiques (Tetlow *et al.*, 2005). Ces derniers peuvent aussi contenir du malate issu de la glycolyse, qui est décarboxylé en pyruvate par l'enzyme malique NADP-dépendante (NADP-ME). Le pyruvate est ensuite transformé par le complexe de la pyruvate déshydrogénase en acétyl-coenzyme A (acétylCoA), le précurseur des acides gras. Ces deux réactions produisent du NADPH et du NADH, respectivement, qui peuvent servir à la synthèse d'acides gras (Tetlow *et al.*, 2005). Enfin, de l'ATP produit par la glycolyse, c'est-à-dire par phosphorylation de substrat (équation [1]), est vraisemblablement consommé lors de biosynthèses telles que celles d'amidon de réserve et d'acides gras dans les plastes non-photosynthétiques. Toutefois, cette contribution n'a pas été quantifiée (Neuhaus et Emes, 2000).

Tableau 1.1 : Voies de biosynthèses alimentées en squelettes carbonés par la glycolyse.

Précurseur glycolytique	Voie alimentée par le précurseur	Références
D-glucose-6-phosphate	Synthèse de polysaccharides pariétaux	Dennis et Blakeley, 2000
	Voie des pentoses-phosphates (cellules non-photosynthétiques) et par suite, voie du shikimate et synthèse de nucléotides	Kruger et von Schaewen, 2003
D-glucose-1-phosphate	Synthèse d'amidon (plastes hétérotrophes)	Neuhaus et Emes, 2000
D-glycéraldéhyde-3-phosphate	Synthèse d'acides aminés	Coruzzi et Last, 2000
dihydroxyacétone-phosphate	Synthèse de glycérol pour les triglycérides	Dennis et Blakeley, 2000
3-D-phosphoglycérate	Synthèse d'acides aminés	Coruzzi et Last, 2000
phospho*énol*pyruvate	Voie du shikimate, impliquée dans la synthèse d'acides aminés aromatiques, alcaloïdes, phénylpropanoïdes	Coruzzi et Last, 2000; Croteau *et al.*, 2000
pyruvate	Synthèse d'acides aminés	Coruzzi et Last, 2000
	Synthèse de terpénoïdes, phénylpropanoïdes	Croteau *et al.*, 2000
	Cycle de Krebs, impliqué dans la synthèse d'acides aminés et, indirectement, d'acides nucléiques et de porphyrines	Dennis et Blakeley, 2000
	Synthèse d'acides gras	Schwender et Ohlrogge, 2002

La complexité du réseau métabolique dans lequel s'inscrit la glycolyse végétale est encore augmentée par son lien étroit avec la néoglucogenèse et l'OPPP. La néoglucogenèse consiste en une décarboxylation de l'OAA en PEP suivie des réactions inverses de la glycolyse du PEP jusqu'au D-saccharose ("sucrose", Suc) (Siedow et Day, 2000). L'existence d'une telle voie anabolique fonctionnant à l'inverse de la glycolyse (voie catabolique), notamment à la germination et à la sénescence, a valu à la glycolyse d'être qualifiée de voie amphibolique (Plaxton, 1996; Siedow et Day, 2000). L'OPPP, quant à elle, partage 3 intermédiaires communs avec la glycolyse : le G6P, le D-fructose-6-phosphate (F6P) et le D-glycéraldéhyde-3-phosphate (G3P) (Tetlow *et al.*, 2005). Des études de marquage de sucres au C^{13} menées dans divers systèmes végétaux ont montré d'importants échanges de carbone entre les trois voies métaboliques, au niveau de leurs intermédiaires communs. Les squelettes carbonés produits par la glycolyse peuvent ainsi parcourir la néoglucogenèse et/ou l'OPPP, ou des portions de celles-ci, avant de réintégrer la glycolyse et de rejoindre les voies biosynthétiques ou de la respiration (Dieuaide-Noubhani *et al.*, 1995; Glawischnig *et al.*, 2002; Schwender et Ohlrogge, 2002; Ettenhuber *et al.*, 2005).

La glycolyse végétale est donc le noyau central d'un réseau de voies de biosynthèses et de voies productrices d'ATP et NAD(P)H qu'elle alimente en squelettes carbonés, ou avec lesquelles elle échange des intermédiaires métaboliques. D'autres caractéristiques de son organisation sont présentées dans le paragraphe suivant, qui confèrent à la glycolyse une flexibilité métabolique elle aussi de prime importance dans le métabolisme végétal.

1.1.2 Structure et flexibilité métabolique de la glycolyse végétale

La glycolyse végétale se distingue de ses équivalents chez *S. cerevisiae* et chez les animaux par sa distribution subcellulaire, sa structure en réseau de réactions redondantes, et par la flexibilité métabolique qui en découle.

1.1.2.1 Compartimentation subcellulaire de la voie

Dans la plupart des types de cellules végétales, la glycolyse a lieu tout entière, ou en partie, dans le cytosol mais aussi dans le plaste (Fig. 1.1) (Plaxton, 1996). Il a été démontré chez certaines espèces que les chloroplastes, les amyloplastes, les leucoplastes ou les chromoplastes de fruits et de pétales peuvent effectuer la séquence complète de réactions de la glycolyse. D'autres espèces comportent des chloroplastes ou des leucoplastes racinaires dépourvus de certaines enzymes glycolytiques (Plaxton, 1996; Tetlow *et al.*, 2005). À l'inverse, plusieurs espèces d'algues unicellulaires sont dotées d'une voie complète dans les chloroplastes mais pas dans le cytosol (Plaxton, 1996). Les réactions se déroulant dans le cytosol et le plaste sont vraisemblablement catalysées par des isoformes encodées par des gènes nucléaires distincts et qui peuvent différer dans leurs propriétés physiques, cinétiques et immunologiques (Plaxton, 1996; Givan, 1999). Les proportions relatives de ces isoformes peuvent varier en fonction du tissu et du stade de développement, ou suite à des changements dans l'environnement ou l'apport en nutriments à la plante (Plaxton, 1996). La nature-même des substrats de la glycolyse et leur origine subcellulaire sont susceptibles de varier selon l'organe considéré. Dans les organes chlorophylliens à la lumière, il s'agit de trioses-phosphates (trioses-Ps) produits dans les chloroplastes par photosynthèse. Dans les organes chlorophylliens à l'obscurité et certains organes de réserve (ex : les tubercules), l'amidon plastidique est hydrolysé en hexoses ou phosphorolysé en hexoses-phosphates (hexoses-Ps). Enfin, les organes hétérotrophes sont approvisionnés via le phloème en Suc dont le catabolisme produit des hexoses et hexoses-Ps dans le cytosol (Fig. 1.1) (Quick et Schaffer, 1996; Smith *et al.*, 2005). Malgré la compartimentation de la glycolyse et de ses sources de substrats, des interactions contrôlées ont lieu entre ses composantes cytosolique et plastidique par l'intermédiaire de transporteurs spécifiques de métabolites (Fig. 1.1). Ceux-ci incluent les translocateurs de triose-phosphate/phosphate inorganique P_i (TPT), du G6P/P_i (GPT) et du PEP/P_i (PPT), un translocateur putatif d'hexose-P/P_i spécifique du D-glucose-1-phosphate (G1P), et des transporteurs putatifs de Glc (pGlcT), de pyruvate et de malate situés dans la membrane interne de l'enveloppe chloroplastique ou plastidique (Tetlow *et al.*, 2005; Weber *et al.*, 2005). Plusieurs d'entre eux ont été impliqués dans des échanges rapides d'intermédiaires glycolytiques entre le cytosol et le plaste dans les pointes

racinaires de maïs (*Zea mays*) et l'embryon de colza (*Brassica napus*) (Dieuaide-Noubhani *et al.*, 1995; Schwender *et al.*, 2003). Dans le cas de la synthèse d'acides gras dans les tissus non-chlorophylliens, ces transporteurs (hormis le TPT et le pGlcT) approvisionnent le plaste en G6P, PEP, pyruvate et malate issus de la glycolyse cytosolique (Neuhaus et Emes, 2000). Par conséquent, leur régulation en fonction de l'espèce et du stade de développement détermine les contributions relatives en intermédiaires glycolytiques du cytosol et du plaste dans la production d'acétylCoA plastidique (Tetlow *et al.*, 2005). La situation est similaire en ce qui concerne l'origine cytosolique ou plastidique de l'ADP-D-glucose (ADP-Glc) pour la synthèse d'amidon dans les plastes hétérotrophes. Selon l'espèce et le type d'organe, ce processus est susceptible d'impliquer les activités du GPT, du translocateur putatif d'hexose-P/P_i spécifique du G1P, ou d'un transporteur putatif d'ADP-Glc (Neuhaus et Emes, 2000; Tetlow *et al.*, 2005). Ces divers transporteurs de métabolites intègrent donc le métabolisme glycolytique du plaste à celui du cytosol malgré la séquestration de la voie (complète ou incomplète), de ses substrats et de ses produits dans les deux compartiments cellulaires. Il s'ensuit une flexibilité métabolique propre à la glycolyse végétale, et qui implique directement la régulation de ses composantes cytosolique et plastidique et des transporteurs considérés ci-dessus.

La glycolyse peut être compartimentée au sein-même du cytoplasme, comme le montre l'attachement de la voie complète et fonctionnelle à la face cytosolique de la membrane externe mitochondriale de suspensions cellulaires d'*Arabidopsis* (Giegé *et al.*, 2003). Un tel arrangement permettrait au pyruvate cytosolique d'être acheminé directement à la mitochondrie, optimisant ainsi l'approvisionnement de cette dernière en substrat respiratoire (Giegé *et al.*, 2003). L'association de la glycolyse à d'autres membranes n'a pas été investigué. Néanmoins, il a été démontré chez l'épinard (*Spinacia oleracea*) qu'une isoforme de l'enzyme glycolytique hexokinase (HK, EC 2.7.1.1, Annexe 1) est ancrée dans la membrane externe de l'enveloppe chloroplastique (Wiese *et al.*, 1999). D'autres sites d'ancrage à des membranes et au cytosquelette ont été suggérés pour cette enzyme qui engage les hexoses dans la glycolyse (Frömmer *et al.*, 2003; Balasubramanian *et al.*, 2005).

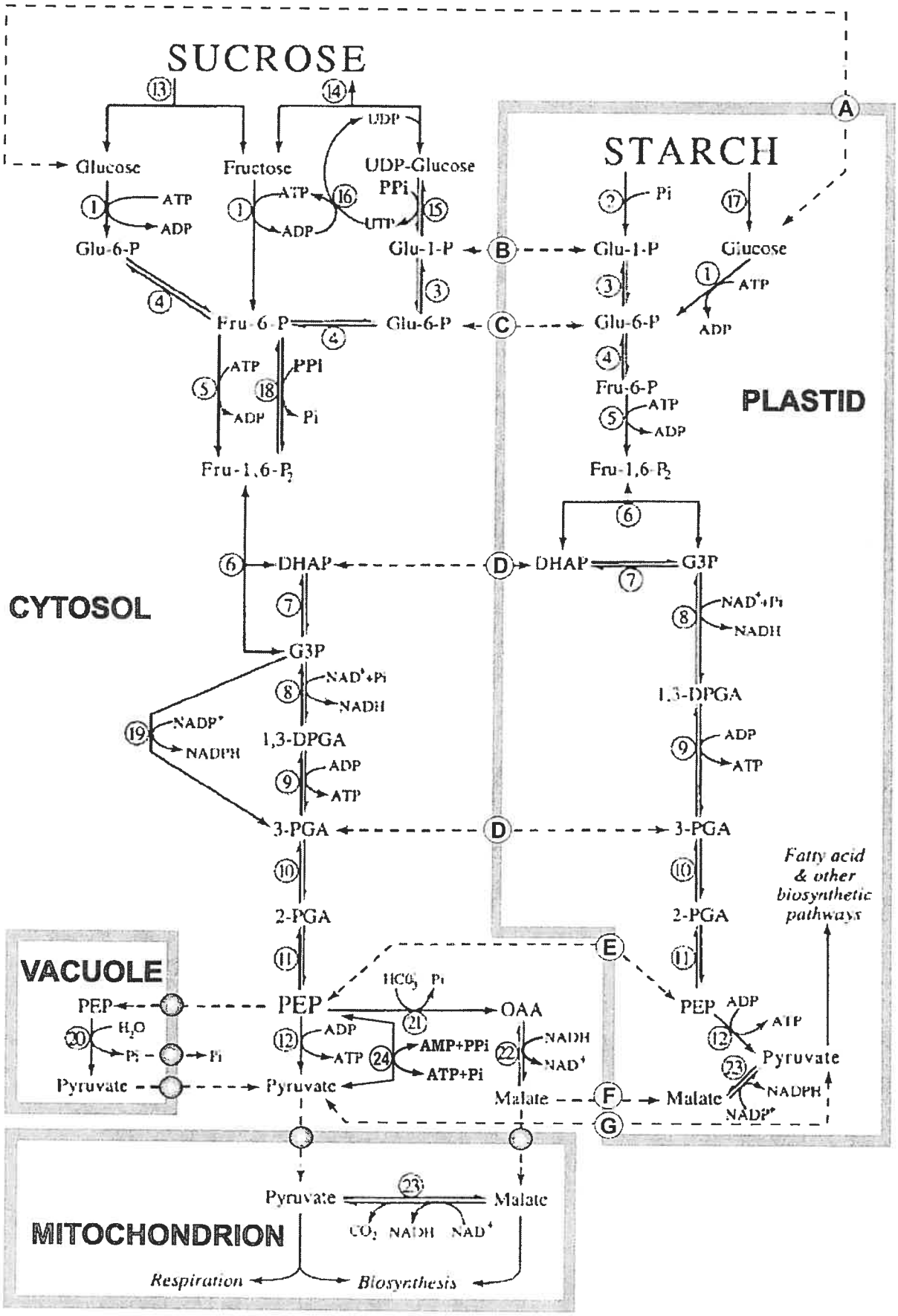
SUCROSE
STARCH
PLASTID
CYTOSOL
VACUOLE
MITOCHONDRION
Glucose
Fructose
UDP-Glucose
UDP
PPi
UTP
ATP
ADP
Pi
Glu-6-P
Glu-1-P
Fru-6-P
Fru-1,6-P2
DHAP
G3P
NAD+ +Pi
NADH
1,3-DPGA
NADP+
NADPH
3-PGA
2-PGA
PEP
H2O
OAA
HCO3- Pi
AMP+PPi
ATP+Pi
Pyruvate
Malate
CO2
Respiration
Biosynthesis
Fatty acid & other biosynthetic pathways
A
B
C
D
E
F
G

Figure 1.1 : Structure et compartimentation de la glycolyse chez les plantes (tiré et adapté de Plaxton, 1996, avec la permission © de Annual Reviews).

Les enzymes glycolytiques sont numérotées comme suit : 1, HK, ou FK si le substrat est le Fru. 2, phosphorylase. 3, PGM. 4, PGI. 5, PFK. 6, ALD. 7, TPI. 8, NAD-GAPDH (P) cytosolique, NAD- et NADP-GAPDH (P) plastidiques. 9, 3-PGA kinase. 10, 3-PGA mutase. 11, énolase. 12, PK. 13, INV. 14, SuSy. 15, UGPase. 16, NDPK. 17, α- et β-amylases. 18, PFP. 19, NADP-GAPDH (NP). 20, PEPase. 21, PEPC. 22, MDH. 23, NAD(P)-ME. 24, PPDK (Plaxton, 1996). Les métabolites 1,3-DPGA, Fru-6-P, Fru-1,6-P_2, Glu-1-P et Glu-6-P sont abrégés 1,3BPGA, F6P, F1,6BP, G1P et G6P dans le texte, respectivement. Les flèches à double sens indiquent des réactions réversibles *in vivo* ; les flèches simples, des réactions irréversibles. Les flèches en pointillés décrivent le passage d'intermédiaires glycolytiques entre différents compartiments subcellulaires. Les transporteurs spécifiques de métabolites sont représentés par des cercles. A, pGlcT. B, translocateur putatif d'hexose-P/P_i spécifique du G1P. C, GPT. D, TPT. E, PPT. F, transporteur de malate. G, transporteur putatif de pyruvate (Tetlow *et al.*, 2005; Weber *et al.*, 2005). Les noms complets des enzymes, métabolites et transporteurs figurent dans la liste des abréviations.

1.1.2.2 Structure de la voie en réseau

La voie cytosolique de la glycolyse végétale se distingue de ses homologues chez *S. cerevisiae* et chez les animaux par des réactions enzymatiques redondantes à plusieurs étapes incluant celles du Suc, du F6P, du G3P et du PEP (Plaxton, 1996). Ainsi, le Suc peut être hydrolysé par l'invertase (INV) en Glc et Fru, qui sont ensuite phosphorylés en G6P et F6P, respectivement, selon deux réactions consommatrices d'ATP catalysées par l'HK (Fig. 1.1). Le Suc peut également être converti par la Suc synthase (SuSy) en Fru et UDP-D-glucose (UDP-Glc) en présence d'UDP, selon une réaction réversible (Fig. 1.1). Le Fru est phosphorylé en F6P par l'HK. L'UDP-Glc, en présence de pyrophosphate inorganique (PP_i), est transformé en UTP et G1P selon une réaction réversible catalysée par l'UDP-D-glucose pyrophosphorylase (UGPase). L'ADP et l'UTP générés sont alors recyclés par la nucléoside-5'-diphosphate kinase (NDPK) en ATP et UDP, respectivement (Fig. 1.1). Par conséquent, la conversion d'1 Suc en 2 hexoses-Ps consomme 2 ATPs via l'INV/HK alors qu'elle consomme 1 PP_i via la SuSy/UGPase. Le PP_i est généré par de nombreuses réactions de biosynthèses sans besoin apparent d'une dégradation d'ATP (Plaxton, 1996). Le flux de carbone par une voie ou par l'autre a donc un impact sur le bilan en ATP de la glycolyse cytosolique. Notons également que lors du catabolisme du Suc, le Fru est phosphorylé par l'HK ou bien par la fructokinase (FK), selon une réaction irréversible et ATP-dépendante (Fig. 1.1). La FK et l'HK sont des enzymes distinctes qui ne partagent pas d'homologie de séquence (Dai *et al.*, 2002). Théoriquement, le recours à différentes enzymes pour catalyser une même étape glycolytique offre des moyens supplémentaires de réguler finement cette étape. Il en va de même pour une étape catalysée par une enzyme présente sous plusieurs isoformes aux caractéristiques uniques de cinétique et de régulation (Plaxton, 1996). Dans le tubercule de pomme de terre (*Solanum tuberosum*), par exemple, les proportions relatives en isoformes FK et HK et leurs propriétés respectives interviennent dans la régulation du métabolisme des hexoses aux différents stades de développement (Renz *et al.*, 1993; Renz et Stitt, 1993). Il ressort que certaines étapes de la dégradation du Suc en hexoses-Ps sont catalysées par différentes isoformes ou par des enzymes distinctes, ce qui étend les possibilités de régulation fine de ces étapes de la glycolyse cytosolique. De

plus, la voie de l'INV/HK et celle de la SuSy/UGPase offrent à la cellule une flexibilité quant au coût en ATP associé à l'entrée des hexoses-Ps dans la voie.

Une seconde étape de la glycolyse cytosolique comportant deux réactions en parallèle est celle du F6P (Fig. 1.1). Ce dernier peut être phosphorylé en D-fructose-1,6-bisphosphate (F1,6BP) par la phosphofructokinase ATP-dépendante (PFK) ou par la PP_i:D-fructose-6-phosphate 1-phosphotransférase (PFP). La PFK consomme 1 ATP tandis que la PFP consomme 1 PP_i et produit 1 P_i par F6P transformé, ce qui ajoute à la flexibilité de la voie vis-à-vis du bilan en ATP. L'étape du G3P est un autre nœud métabolique qui implique deux isoformes cytosoliques de la G3P déshydrogénase (GAPDH) : la GAPDH $NADP^+$-dépendante, non phosphorylante (NADP-GAPDH [NP]) et la GAPDH NAD^+-dépendante, phosphorylante (NAD-GAPDH [P]) (Rivoal *et al.*, 2004). La réaction réversible de la NADP-GAPDH (NP) produit du 3-phospho-D-glycérate (3-PGA) en parallèle à celles de la NAD-GAPDH (P) et de la 3-PGA kinase (Fig. 1.1). La NADP-GAPDH (NP) consomme 1 $NADP^+$ au lieu d'1 NAD^+ + 1 P_i + 1 ADP par la NAD-GAPDH (P)/3-PGA kinase. Outre la possibilité de régulation fine par le biais de plusieurs isoformes GAPDH, la consommation d'ADP et P_i peut être réduite à cette étape si le flux glycolytique passe par la NADP-GAPDH (NP). D'une façon comparable, à l'étape du PEP les réactions de la PEP phosphatase (PEPase), de la PEP carboxylase (PEPC) et de la pyruvate, phosphate dikinase (PPDK) sont des alternatives à celle de la pyruvate kinase (PK) consommatrice d'ADP (Fig. 1.1). La PEPase et la PPDK produisent du pyruvate selon deux réactions distinctes, la première étant irréversible et la seconde, réversible. La PEPC carboxyle le PEP en OAA, qui est transformé en malate par la malate déshydrogénase (MDH) puis en pyruvate par la NAD-ME. La PEPase et la PEPC produisent chacune 1 P_i par PEP transformé, tandis que la PPDK consomme 1 AMP + 1 PP_i.

La structure en réseau de la glycolyse cytosolique lui confère donc une capacité de régulation fine de certaines étapes du fait de leur catalyse par des enzymes distinctes. Cette capacité est encore augmentée par l'expression de plusieurs enzymes sous différentes isoformes. Par ailleurs, les étapes du Suc, F6P, G3P et PEP comportent des réactions alternatives à celles de la voie linéaire classique consommatrices d'ATP, d'ADP ou de P_i. La flexibilité métabolique qui découle de cette structure en réseau peut faciliter le

développement de la plante, mais également jouer un rôle déterminant dans sa réponse adaptative à certains stress (Plaxton, 1996). Cet aspect de la glycolyse végétale est décrit plus amplement ci-dessous.

1.1.3 Rôle de la glycolyse dans la réponse des plantes aux stress abiotiques

1.1.3.1 La glycolyse est un élément majeur des adaptations métaboliques des plantes aux stress abiotiques

Les plantes sont inévitablement soumises à des changements dans leur environnement physico-chimique qui sont à l'origine de stress dits abiotiques (Smirnoff, 1995; Rivoal *et al.*, 2004). Ces stress, tels un apport inadéquat en eau, lumière, O_2 ou en certains nutriments, affectent la croissance et la distribution géographique des plantes sauvages et limitent la productivité des plantes cultivées (Boyer, 1982; Bray *et al.*, 2000). À titre d'exemple, les rendements à l'hectare des huit plantes les plus cultivées aux États-Unis en 1965 n'atteignaient en moyenne que 30% des chiffres record enregistrés, les pertes de rendement étant attribuées aux seuls stress abiotiques (Boyer, 1982). Par conséquent, la sélection de variétés mieux adaptées à des sols et climats défavorables peut aboutir à de larges gains de productivité, mais nécessite d'abord de mieux comprendre les mécanismes impliqués dans la réponse adaptative aux stress abiotiques (Boyer, 1982). Ceux-ci peuvent être de type développemental, structural, physiologique ou métabolique (McCue et Hanson, 1990). La glycolyse apparaît comme un élément majeur des adaptations métaboliques des plantes à divers stress abiotiques (Smirnoff, 1995; Rivoal *et al.*, 2004). Un argument en faveur d'une telle implication est que presque toutes les enzymes glycolytiques voient leurs niveaux d'activité, de protéines, ou d'ARNms correspondants augmenter en réponse à un ou plusieurs stress abiotiques (Vartapetian *et al.*, 2003; Rivoal *et al.*, 2004). C'est le cas de la grande majorité des enzymes glycolytiques dans divers organes végétaux exposés au manque d'O_2 ou de P_i (Tableau 1.2) (Rivoal *et al.*, 2004, et sources citées). C'est aussi le cas de plusieurs d'entre elles dans la réponse adaptative d'organes non-photosynthétiques à une carence en azote ou en fer, au froid ou au stress salin (Wang *et al.*, 2003; Rivoal *et al.*, 2004; Yan *et al.*, 2005). La réponse biochimique et moléculaire des plantes à un apport

insuffisant en O_2 (anaérobiose) nous sert de cas d'étude ci-dessous afin d'illustrer en quoi consiste le rôle de la glycolyse dans la réponse adaptative aux stress abiotiques.

Tableau 1.2 : Effet d'un apport insuffisant en O_2 sur les niveaux d'ARNms, de protéines ou d'activité d'enzymes glycolytiques chez diverses espèces végétales (adapté de Rivoal *et al.*, 2004).

Enzyme, espèce, matériel végétal, niveau d'activité, de protéine, ou d'ARNm correspondant	Traitement hypoxique, anoxique, ou anaérobie [a] : facteur d'induction	Référence
D-saccharose synthase (SuSy)		
O. sativa, plantules, activité	5 j d'anoxie : 2,3	[a]
protéine et ARNm	24 h d'anoxie : i.s. [b]	[a]
Z. mays, racines, activité	24 h d'hypoxie à 3% O_2 : 4	[b]
ARNm	6 h d'anaérobiose : 13	[c]
tiges, ARNm	6 h d'anaérobiose : 7	[c]
fructokinase (FK)		
T. subterraneum, racines, activité	36 h d'hypoxie à 3% O_2 : 2,2	[d]
A. thaliana, plantes, ARNm	24 h d'hypoxie à 3% O_2 : i.s.	[e]
racines, ARNm	20 h d'hypoxie à 5% O_2 : i.s.	[f]
hexokinase (HK)		
E. crus-pavonis, racines, activité	2 j d'anoxie : 3	[g]
E. phyllopogon, racines, activité	2 j d'anoxie : 9	[g]
tiges, activité	2 j d'anoxie : 5	[g]
O. sativa, tiges, activité	8 j d'anoxie : 3	[h]
Z. mays, racines, activité	16 h d'hypoxie à 3% O_2 : > 4	[i]
A. thaliana, plantules, ARNm	6 h d'anoxie : i.s.	[j]
phosphoglucomutase (PGM)		
O. sativa, plantules, activité	24 h d'anoxie : 1,4	[k]
Z. mays, racines, activité	72 h d'anoxie : 2	[l]
A. thaliana, plantules, ARNm	6 h d'anoxie : i.s.	[j]
phosphoglucose isomérase (PGI)		
O. sativa, plantules, activité	24 h d'anoxie : 1,7	[k]
Z. mays, racines, activité	72 h d'anoxie : 2,2	[l]
A. thaliana, plantules, ARNm	6 h d'anoxie : i.s.	[j]
PP_i-D-fructose-6-phosphate 1-phosphotransférase (PFP)		
O. sativa, coléoptyles, activité	5 j d'anoxie : i.s.	[m]
Z. mays, plantules, activité	8 j d'anoxie : i.s.	[n]
A. thaliana, plantules, ARNm	6 h d'anoxie : i.s.	[j]

aldolase (ALD)		
O. sativa, plantules, activité	24 h d'anoxie : 1,9	[k]
Z. mays, racines de plantules, activité	72 h d'anoxie : 1,3	[l]
Z. mays, racines acclimatées, ARNm	12 h d'anoxie : 6	[o]
tiges acclimatées, ARNm	12 h d'anoxie : 2,5	[o]
D-glycéraldéhyde-3-phosphate déshydrogénase NAD-dépendante, phosphorylante (NAD-GAPDH [P])		
O. sativa, plantules, activité	72 h d'anoxie : 3,6	[k]
plantules, ARNm	24 h d'anoxie : i.s.	[k]
Z. mays, racines de plantules, activité	72 h d'anoxie : 1,5	[l]
pointes racinaires, protéine	4 h d'hypoxie à 3% O_2 : i.s.	[p]
racines, ARNm	6 h d'anaérobiose : 6	[c]
tiges, ARNm	6 h d'anaérobiose : 15	[c]
énolase		
E. crus-pavonis, tiges, activité	12 h d'anoxie : 5	[q]
Z. mays, racines, protéine	4 h d'hypoxie à 3% O_2 : i.s.	[p]
racines acclimatées, ARNm	6 h d'hypoxie à 4% O_2 : 2,4	[o]
tiges acclimatées, ARNm	24 h d'anoxie : 2	[o]
pyruvate, phosphate dikinase (PPDK)		
O. sativa, plantules, activité	72 h d'anoxie : 1,4	[r]
racines, protéine	72 h d'anoxie : i.s.	[r]
coléoptyles, protéine	72 h d'anoxie : > 5	[s]
ARNm	12 h d'anoxie : i.s.	[r]
malate déshydrogénase (MDH)		
Z. mays, racines, activité	72 h d'anoxie : 1,2	[l]
O. sativa, plantules, activité	72 h d'anoxie : 1,3	[r]

[a] Voir les définitions d'hypoxie et anoxie dans le texte. Le terme anaérobie décrit ici un degré d'hypoxie variable dans le temps et pouvant évoluer vers l'anoxie du fait de basses concentrations d'O_2 non contrôlées.

[b] i.s., induction significative.

[a] Ricard *et al.*, 1991; [b] Zeng *et al.*, 1998; [c] Russell et Sachs, 1989; [d] Aschi-Smiti *et al.*, 2003; [e] Liu *et al.*, 2005; [f] Klok *et al.*, 2002; [g] Fox *et al.*, 1998; [h] Guglielminetti *et al.*, 1995; [i] Bouny et Saglio, 1996; [j] Loreti *et al.*, 2005; [k] Rivoal *et al.*, 1989; [l] Kelley et Freeling, 1984; [m] Gibbs *et al.*, 2000; [n] Mertens *et al.*, 1990; [o] Andrews *et al.*, 1994; [p] Chang *et al.*, 2000; [q] Fox *et al.*, 1995; [r] Moons *et al.*, 1998; [s] Huang *et al.*, 2005.

1.1.3.2 Cas d'étude : le rôle de la glycolyse dans la réponse au stress anaérobie

Les végétaux sont des organismes aérobies obligatoires : l'O_2 leur est indispensable en tant qu'accepteur final d'électrons durant la phosphorylation oxydative, qui produit l'essentiel de l'ATP cellulaire. Pour cette raison, leur survie peut être compromise en conditions anaérobies, c'est-à-dire lorsqu'un apport insuffisant en O_2 de l'air limite ou même bloque l'activité respiratoire (Bray *et al.*, 2000). L'anaérobiose survient dans divers organes végétaux dont l'épaisseur, la densité ou le tégument limite la diffusion de l'O_2 atmosphérique, comme certains fruits, graines et tubercules (Geigenberger, 2003). Les graines et les racines sont également exposées à ce stress lorsque le sol est saturé d'eau ou inondé, étant donné que l'O_2 de l'air y diffuse jusqu'à 10^4 fois plus lentement que dans un sol bien drainé (Drew, 1997). Les sols anaérobies concernent aussi bien des écosystèmes naturels (ex : marécages subtropicaux de Floride) que des cultures en champs inondés (ex : rizières) ou des cultures irriguées et mal drainées (ex : régions sud, est et ouest de l'Australie). Il s'agit ici de millions d'hectares répartis dans diverses régions du globe (Gibbs et Greenway, 2003). Il semble donc que l'anaérobiose soit un phénomène largement répandu dans le règne végétal (Geigenberger, 2003; Rivoal *et al.*, 2004), et la réponse métabolique à ce stress a été caractérisée dans divers organes et tissus.

Au niveau cellulaire, la privation d'O_2 restreint ou même inhibe la respiration, ce qui entraîne une crise du rendement énergétique. En effet, l'oxydation complète d'1 hexose par la respiration produit 30–36 ATPs, contre seulement 2–3 ATPs par la glycolyse (Gibbs et Greenway, 2003). En réponse à cette crise, de nombreuses voies biosynthétiques sont réprimées, réduisant ainsi la demande en ATP (Geigenberger, 2003; Gibbs et Greenway, 2003). Il peut s'ensuivre également une augmentation du flux glycolytique, appelée effet Pasteur, qui compense partiellement le faible rendement en ATP de la voie et minimise le déficit énergétique (Geigenberger, 2003; Gibbs et Greenway, 2003). La valeur adaptative de l'effet Pasteur dans la survie en anaérobiose est largement acceptée dans la littérature (Geigenberger, 2003; Gibbs et Greenway, 2003; Vartapetian *et al.*, 2003; Rivoal *et al.*, 2004). La hausse du flux glycolytique ne peut avoir lieu sans un ajustement dans l'apport en substrats, le recyclage du NADH en NAD^+ et l'élimination des produits finaux de la glycolyse (Gibbs et Greenway, 2003). Le dernier point est important car en s'accumulant,

les produits du métabolisme anaérobie sont susceptibles d'inhiber l'activité glycolytique, par rétrocontrôle négatif par exemple (Gibbs et Greenway, 2003). De plus, certains d'entre eux sont potentiellement cytotoxiques, comme l'acétaldéhyde ou le lactate (Gibbs et Greenway, 2003). En conditions anaérobies, le recyclage du NADH en NAD^+ est assuré principalement par la fermentation éthylique ; la contribution de la respiration est négligeable, voire nulle (Ricard *et al.*, 1994; Gibbs et Greenway, 2003). La glycolyse couplée à la fermentation éthylique est alors la principale source d'ATP en l'absence totale d'O_2 (anoxie) puisque la respiration est inhibée. Cette situation diffère de l'hypoxie où la respiration et la fermentation coexistent dans des proportions qui dépendent de la concentration d'O_2 cellulaire et de l'apport en hexoses (Ricard *et al.*, 1994). Hormis la fermentation éthylique, d'autres systèmes recyclent le NADH en NAD^+ en anaérobiose, comme les réactions inverses d'une portion du cycle de Krebs qui génèrent du succinate et de l'acide γ-aminobutyrique (GABA) à partir d'OAA. Citons également les réactions de fermentation lactique et de l'Ala, si cette dernière est couplée à la réduction du nitrate NO_3^- (Ricard *et al.*, 1994; Gibbs et Greenway, 2003). En hypoxie, la réduction du NO_3^- consomme du NADH et génère de l'oxyde nitrique. La détoxification de ce dernier impliquerait son oxydation par l'hémoglobine non-symbiotique, selon une réaction elle-même consommatrice de NADH (ou NADPH) (Igamberdiev *et al.*, 2005). À des degrés divers, les systèmes évoqués ci-dessus peuvent donc contribuer au recyclage du NADH en anaérobiose, autorisant ainsi une hausse de l'activité glycolytique génératrice d'ATP (Ricard *et al.*, 1994; Gibbs et Greenway, 2003; Rivoal *et al.*, 2004; Igamberdiev *et al.*, 2005). Enfin, il se peut que l'effet Pasteur requière l'induction significative de plusieurs enzymes glycolytiques pour avoir lieu (Tableau 1.2). Ainsi, l'induction concertée de plusieurs enzymes glycolytiques aux niveaux transcriptionnel et posttranscriptionnel a été interprétée comme un moyen pour la cellule d'élever le taux de production d'ATP en anaérobiose (Fennoy *et al.*, 1998). Cette hypothèse s'accorde avec l'argument selon lequel une voie métabolique nécessite l'activation de plusieurs de ses enzymes pour voir son flux augmenter fortement et rapidement, sans perturber les concentrations en intermédiaires (Small et Kacser, 1993; Fell et Thomas, 1995).

Puisque l'anaérobiose engendre une crise du rendement énergétique du métabolisme primaire, la flexibilité de la glycolyse vis-à-vis de son bilan en ATP devient un enjeu majeur dans la réponse adaptative à ce stress (Plaxton, 1996; Rivoal *et al.*, 2004). Rappelons que les étapes ATP-dépendantes de l'INV/HK et de la PFK peuvent être court-circuitées par les réactions PP_i-dépendantes de la SuSy/UGPase et de la PFP, respectivement (Fig. 1.1). Or en anaérobiose, les niveaux d'ATP tendent à décliner alors que le PP_i cytosolique demeure à des concentrations élevées et stables, de l'ordre de 0,3 mM (Plaxton, 1996). De plus, en anaérobiose l'énergie libre d'hydrolyse du PP_i augmente (en valeur absolue) tandis que celle de l'ATP diminue, ce qui favorise le PP_i plutôt que l'ATP comme donneur de groupement phosphoryl (Davies *et al.*, 1993). En l'absence apparente de consommation d'ATP pour sa biosynthèse, le PP_i a donc été proposé comme une alternative à l'ATP comme donneur d'énergie en anaérobiose (Plaxton, 1996). Par conséquent, une redirection du flux glycolytique via la SuSy/UGPase et la PFP en anaérobiose réduit le coût en ATP à deux étapes de la voie et améliore son rendement énergétique (Plaxton, 1996; Rivoal *et al.*, 2004). En accord avec cette hypothèse, l'induction d'expression et d'activité PFP et SuSy, et l'inhibition d'expression et d'activité INV en conditions anaérobies, ont été largement documentées (Tableau 1.2) (Plaxton, 1996; Geigenberger, 2003; Rivoal *et al.*, 2004). Par ailleurs, la réaction de la PPDK, qui génère de l'ATP à partir d'AMP et de PP_i, est plus efficace d'un point de vue énergétique que la réaction alternative de la PK, qui produit de l'ATP à partir d'ADP et de P_i (Fig. 1.1) (Rivoal *et al.*, 2004). L'induction de la PPDK en anaérobiose est donc susceptible d'améliorer encore le rendement énergétique de la glycolyse (Tableau 1.2) (Rivoal *et al.*, 2004).

La réponse métabolique des plantes au stress anaérobie consiste donc à résoudre une crise du rendement énergétique (Gibbs et Greenway, 2003). La flexibilité de la glycolyse vis-à-vis de son coût en ATP, et la hausse de son activité productrice d'ATP, sont deux éléments majeurs de cette réponse adaptative (Plaxton, 1996). De même, la réponse à une carence du milieu en P_i implique la flexibilité de la glycolyse quant à son bilan en ATP, ADP et P_i, et une hausse de son flux vers la production d'acides organiques (Theodorou et Plaxton, 1993; Rivoal *et al.*, 2004). Ajoutons que la réponse des plantes au froid, ou à un

appauvrissement du milieu en fer, repose elle aussi sur la flexibilité métabolique de la glycolyse et sur une hausse de son flux (Rivoal *et al.*, 2004).

Ces données finissent donc d'illustrer l'adéquation entre l'organisation de la glycolyse et ses multiples rôles dans le métabolisme végétal. Ainsi, nous avons vu que la glycolyse végétale est connectée à un vaste réseau de voies métaboliques qu'elle alimente en squelettes carbonés (Plaxton, 1996). Elle se distingue aussi par sa compartimentation dans le cytosol et le plaste, et par sa structure en réseau de réactions redondantes (Plaxton, 1996). De ces deux caractéristiques découle la flexibilité métabolique de la glycolyse, qui facilite le développement de la plante et sa réponse adaptative à divers stress abiotiques. En particulier, le bilan de la voie en ATP, ADP, et P_i, varie selon que son flux parcourt certaines de ses portions cytosoliques et plastidiques, ou certaines réactions redondantes plutôt que d'autres (Plaxton, 1996; Rivoal *et al.*, 2004). La hausse du flux glycolytique est également un élément clé de la réponse adaptative des plantes à plusieurs stress (Rivoal *et al.*, 2004). Il est alors essentiel pour notre compréhension de la glycolyse végétale, d'élucider les mécanismes impliqués dans sa régulation et dans le contrôle de son flux.

1.2 Régulation de la glycolyse et contrôle de son flux

La partie précédente décrivait la structure particulière de la glycolyse et montrait en quoi celle-ci est adaptée aux rôles de la voie dans le métabolisme végétal. La question de la régulation de la voie et du contrôle de son flux n'a été abordée que succinctement, au sujet de la réponse aux stress abiotiques. Elle est toutefois indissociable de l'adaptation de la glycolyse à chacun de ses rôles dans le métabolisme, et constitue l'une des clés de notre compréhension de la biochimie de cette voie. Par exemple, la glycolyse a été présentée comme un pilier de l'architecture métabolique de la cellule végétale, en raison de la stabilité de son flux par rapport à l'influx de substrat (Rontein *et al.*, 2002; Spielbauer *et al.*, 2006). Ces résultats soulèvent alors la question de l'efficacité de régulation de la voie. Il importe aussi de reconsidérer les mécanismes de contrôle du flux glycolytique, ce dernier ayant été rarement perturbé par les manipulations d'enzymes jugées "limitantes" (ex : PFK, PK) dans l'analyse métabolique classique (Stitt et Sonnewald, 1995; Fell, 1997). La carte, aussi

complète soit-elle, des réactions composant la glycolyse ne nous renseigne en rien sur la flexibilité de son flux face aux variations de l'environnement (Fell, 1997). Cette partie traite donc de la régulation de la glycolyse et du contrôle de son flux, ce qui nous amène d'abord à distinguer les termes "régulation" et "contrôle". La notion de régulation s'applique à un système métabolique qui maintient une variable relativement constante au cours du temps, en dépit de fluctuations dans l'environnement de ce système (Fell, 1997). Dans certains cas, la régulation peut désigner la modulation du flux d'une voie métabolique suite à une variation des conditions externes, tandis que les niveaux d'intermédiaires restent inchangés. Ce concept s'approche alors de celui d'homéostasie, qui décrit le maintien d'un état interne relativement constant (Fell, 1997). Dans d'autres cas, la régulation peut désigner le maintien d'un flux constant (ex : taux de production d'ATP) malgré les fluctuations dans l'environnement de la voie (Hofmeyr et Cornish-Bowden, 1991). Il ressort que l'efficacité de régulation d'une voie métabolique est relative à la fonction que nous assignons à cette voie (Hofmeyr et Cornish-Bowden, 1991). La notion de contrôle métabolique, quant à elle, décrit la capacité à changer l'état stationnaire d'un système, par exemple le flux d'une voie métabolique. Cette notion n'implique donc pas de préconception à propos de la fonction de la voie considérée (Hofmeyr et Cornish-Bowden, 1991). Maintenant qu'elles sont définies, les notions de régulation et de contrôle peuvent être considérées à propos de la glycolyse.

1.2.1 Régulation de la glycolyse

L'intensité et le parcours du flot de carbone dans la voie glycolytique résultent des activités *in vivo* des enzymes de la voie, qui sont soumises à divers mécanismes de régulation fine ou brute (Plaxton, 1996). La régulation brute affecte la population de molécules d'enzyme dont elle module les taux de synthèse et/ou de dégradation. Elle s'étale sur plusieurs heures ou jours, et intervient notamment lors de la différenciation des tissus ou d'adaptations métaboliques à des changements à long-terme dans l'environnement (Plaxton, 1996; Fell, 1997). La régulation brute de la PFP, la 3-PGA mutase, l'énolase, la PK et la PEPC, a été étudiée aux niveaux transcriptionnel et traductionnel (Plaxton, 1996). En revanche, notre connaissance de la régulation protéolytique des enzymes de la glycolyse reste limitée (Plaxton, 1996; Fernie *et al.*, 2004). Une régulation brute concertée de

plusieurs enzymes glycolytiques a été observée lors de la réponse à l'anaérobiose et lors du développement de la graine (Fennoy *et al.*, 1998; Ruuska *et al.*, 2002; Baud et Graham, 2006).

La régulation fine module l'activité d'une enzyme préexistante sur une échelle de temps de quelques minutes, secondes ou millisecondes (Fell, 1997). Dans le cas des enzymes glycolytiques, elle peut impliquer une variation du pH, une variation de concentration d'un substrat, produit ou effecteur (activateur ou inhibiteur), ou une modification covalente réversible (Plaxton, 1996). La caractérisation cinétique d'enzymes purifiées de PFK, PEPC et PK a permis d'élaborer un schéma de régulation de la glycolyse par ces enzymes et leurs effecteurs respectifs. Ainsi, les PFKs végétales sont particulièrement sensibles au rapport $[P_i]/[PEP]$, l'activateur P_i réduisant l'effet inhibiteur du PEP (Häusler *et al.*, 1989; Vella et Copeland, 1993; Plaxton, 1996). Une hausse de la demande en PEP par les enzymes de la glycolyse et de la voie du shikimate, a donc pour effet de stimuler l'activité PFK en amont de la voie. Ce mode de régulation, dit "bottom up", contraste avec le mode "top down" de la glycolyse animale où le F1,6BP, généré en amont de la voie, régule la PK en aval (Plaxton, 1996). Chez les plantes, l'activité PEPC joue un rôle essentiel dans la boucle régulatrice car elle consomme du PEP et produit du P_i, ce qui fait augmenter le rapport $[P_i]/[PEP]$ et active la PFK (Fig. 1.1). La PEPC est inhibée par plusieurs intermédiaires du cycle de Krebs. Leur consommation dans les voies de biosynthèses connectées au cycle, ou durant la respiration, active donc la PEPC et, indirectement, la PFK (Schuller *et al.*, 1990; Podestá et Plaxton, 1994; Law et Plaxton, 1995). Un effet similaire peut avoir lieu lors de l'accumulation de G6P, ce produit de l'HK étant un activateur de la PEPC (Schuller *et al.*, 1990; Podestá et Plaxton, 1994; Law et Plaxton, 1995). Il ressort que l'activité PEPC peut intégrer la demande en PEP et composés du cycle de Krebs, ainsi que l'apport en intermédiaires en amont de la glycolyse sous forme de G6P, et réguler à son tour la PFK. La PEPC semble donc jouer un rôle dans la coordination de l'activité glycolytique à l'apport en substrats et à la demande en produits de la voie. D'une façon similaire, l'inhibition de la PEPC et de la PK par les acides aminés Asp et Glu a été interprétée comme un moyen de réguler la production par la glycolyse de squelettes carbonés destinés à l'assimilation du NH_4^+ (Huppe et Turpin, 1994). Enfin, la

régulation fine de la glycolyse fait partie intégrante de son rôle dans la réponse des plantes aux stress abiotiques. Par exemple, la baisse des niveaux cytosoliques de P_i suite à sa raréfaction dans l'environnement, active la PFP et la PEPase et inhibe la PFK cytosolique (Plaxton, 1996). Ceci limite le coût de la glycolyse en ATP, ADP et P_i, dont la valeur adaptative en situation de carence en P_i a été mentionnée plus haut. Ces données cinétiques et métaboliques ont contribué à établir la PFK, la PEPC et la PK comme les enzymes régulatrices de la glycolyse végétale (Stitt et Sonnewald, 1995; Plaxton, 1996).

1.2.2 Contrôle du flux glycolytique

L'ATP semble occuper une place particulière dans la régulation de la glycolyse végétale et dans le contrôle de son flux. Les PFKs, PFPs, PEPCs et PKs de plantes ne sont généralement pas inhibées par l'ATP ou l'AMP, alors que ceux-ci occupent une place prépondérante dans la régulation de la glycolyse animale (Plaxton, 1996). En revanche, plusieurs auteurs ont proposé la consommation d'ATP par certains cycles de substrats comme moteur du flux glycolytique chez les plantes (Urbanczyk-Wochniak *et al.*, 2003; Alonso *et al.*, 2005). Un cycle de substrat (appelé auparavant "cycle futile") est composé d'au moins deux réactions distinctes dont l'équation-bilan se résume à une consommation d'énergie, généralement sous forme d'ATP (Portais et Delort, 2002). De tels cycles ont été identifiés au sein de la glycolyse, incluant les cycles du Suc et celui du Glc/Glc-P (Dieuaide-Noubhani *et al.*, 1995; Alonso *et al.*, 2005). Les cycles du Suc impliquent la dégradation de Suc par la SuSy, l'INV et l'HK (ou la FK), et sa recomposition par la SuSy (réaction inverse), la D-saccharose-phosphate synthase (SPS) et la D-saccharose-phosphate phosphatase (Nguyen-Quoc et Foyer, 2001). D'après des études dans divers systèmes végétaux, les cycles du Suc consommaient 5–80% de l'ATP cellulaire (Hill et ap Rees, 1994; Dieuaide-Noubhani *et al.*, 1995; Fernie *et al.*, 2002; Rontein *et al.*, 2002; Alonso *et al.*, 2005). L'expression dans des tubercules de pommes de terre transgéniques d'enzymes catabolisant le Suc ou isomérisant le Fru et le Glc, a entraîné une hausse des flux cycliques du Suc et de l'activité glycolytique (Trethewey *et al.*, 1999; Fernie *et al.*, 2002; Urbanczyk-Wochniak *et al.*, 2003). Il a été conclu que les cycles du Suc interviennent dans le contrôle du flux glycolytique en modulant la demande en ATP, même si d'autres facteurs

indéterminés semblent impliqués (Urbanczyk-Wochniak *et al.*, 2003; Junker *et al.*, 2006). Le cycle du Glc/Glc-P implique l'HK consommatrice d'ATP qui phosphoryle le Glc en G6P, lequel peut être transformé en G1P par la phosphoglucomutase (PGM) (Fig. 1.1). Le Glc-P, c'est-à-dire le G1P ou le G6P, sert ensuite de substrat à une Glc-P phosphatase non identifiée qui reconstitue du Glc (Alonso *et al.*, 2005). Il a été montré que ce cycle consommait jusqu'à 40% de l'ATP dans des pointes racinaires de maïs, tandis que les cycles du Suc n'en consommaient que 6% (Alonso *et al.*, 2005). Par analogie aux cycles du Suc, ces auteurs ont proposé que la variation du flux cyclique du Glc/Glc-P module la demande en ATP cellulaire et participe ainsi au contrôle de l'activité glycolytique. Une telle importance de ces cycles dans le contrôle du flux de la glycolyse expliquerait que leurs flux soient maintenus constants par rapport à l'influx de Glc, y compris en hypoxie (Roscher *et al.*, 1998; Rontein *et al.*, 2002). De plus, cette hypothèse s'accorde avec le rôle démontré de la demande en ATP dans le contrôle du flux glycolytique chez les plantes (Sweetlove *et al.*, 2002). Les cycles du Suc et du Glc/Glc-P seraient donc une variante végétale parmi les divers mécanismes de contrôle du flux glycolytique par l'ATP observés chez les bactéries, levures, plantes et animaux (Thomas et Fell, 1998; Larsson *et al.*, 2000; Koebmann *et al.*, 2002; Fernie *et al.*, 2004; Liu *et al.*, 2006).

Le contrôle du flux glycolytique chez les plantes n'a été quantifié que pour quelques enzymes de la voie, et reste donc moins bien étudié que pour la photosynthèse, la respiration ou la conversion Suc-amidon (Thomas *et al.*, 1997; Poolman *et al.*, 2000; Affourtit *et al.*, 2001; Geigenberger *et al.*, 2004). Estimer le contrôle d'une enzyme sur le flux glycolytique revient à apprécier dans quelle mesure le flux change suite à une variation fractionnelle d'activité de cette enzyme (Fell, 1997). Or, celle-ci est liée à la voie par ses substrat(s), produit(s) ou effecteur(s) qu'elle partage avec d'autres enzymes glycolytiques (Fell, 1997). Le contrôle de l'enzyme sur le flux intègre donc une influence du reste de la voie et nécessite d'être évalué *in situ*, et non sur la base des seules propriétés cinétiques de l'enzyme obtenues hors du contexte cellulaire (Fell, 1997). Ce point constitue une critique majeure de l'analyse métabolique classique, qui assigne le contrôle d'une voie métabolique à une seule enzyme jugée "limitante" d'après ses propriétés cinétiques *in vitro* (Blackman, 1905). La candidate idéale selon l'approche classique est une enzyme en début de voie,

hautement régulée, qui catalyse une réaction irréversible ou présumée loin de l'équilibre, à un taux en deçà de sa V_{max} (Fell, 1997). Ces critères uniquement qualitatifs ont amené à désigner tour à tour la PFK, la PFP et la PK comme l'enzyme limitante de la glycolyse, non sans confusion entre les notions de régulation et de contrôle de flux (Stitt et Sonnewald, 1995). Néanmoins, une large modification de leur activité dans des transformants n'a pas eu d'effet significatif sur le flux glycolytique, la respiration ou la croissance, prouvant que l'approche classique était infondée (Gottlob-McHugh *et al.*, 1992; Burrell *et al.*, 1994; Hajirezaei *et al.*, 1994; Thomas *et al.*, 1997). D'autre part, plusieurs théories émises au cours des années 1970 ont démontré que les principes de l'analyse classique étaient erronés. Parmi celles-ci figure l'Analyse de Contrôle Métabolique (MCA) qui a été amplement développée depuis (Kacser et Burns, 1973; Heinrich et Rapoport, 1974; Fell, 1997), et sur laquelle s'appuie la présente thèse.

La MCA établit que dans un système métabolique, le contrôle du flux d'une voie est réparti sur toutes ses enzymes et sur celles d'autres voies auxquelles elle est connectée via des intermédiaires ou des effecteurs (Kacser *et al.*, 1995). La contribution d'une enzyme E_i au contrôle du flux J est quantifiée par son coefficient de contrôle de flux (FCC), C^J_{Ei}, déterminé expérimentalement d'après la relation suivante :

$$C^J_{Ei} = d (\ln J) / d (\ln v_i) \qquad (1.2)$$

Ceci implique de faire changer l'activité spécifique v_i de E_i par petits incréments, et de mesurer le flux J pour chacun des niveaux d'activité atteints. Seule v_i est autorisée à varier, les autres activités du système métabolique doivent rester intactes. C^J_{Ei} est alors égal à la pente de la tangente à la courbe représentant ln J en fonction de ln v_i (Fig. 1.2) (Kacser *et al.*, 1995; Fell, 1997). Par ailleurs, ces auteurs ont énoncé le théorème de la sommation, selon lequel la somme des n FCCs des enzymes E_1 à E_n participant au contrôle de la voie est égale à 1 :

$$\Sigma_{(i=1 \text{ à } n)} C^J_{Ei} = 1 \qquad (1.3)$$

Par conséquent, une valeur m du coefficient C^J_{Ei} proche de 1 signifie que l'enzyme E_i contribue largement au contrôle du flux J dans les conditions expérimentales établies (Kacser *et al.*, 1995). Le reste du contrôle (1-m) est alors partagé entre les autres enzymes

faisant partie de la voie ou interagissant avec elle. Toutefois, le contrôle du flux J n'est pas réparti uniformément entre toutes ces enzymes, et plusieurs peuvent avoir un FCC nul ou proche de zéro (Fell, 1997). Une conséquence importante du théorème de la sommation est qu'une modification de C^{J}_{Ei} entraîne forcément la modification d'autres FCCs de sorte que leur somme reste égale à 1. Ceci démontre que C^{J}_{Ei} est une propriété du système métabolique autant que de l'enzyme E_i, qui incorpore l'influence des autres enzymes sur E_i dans un état métabolique donné (Kacser *et al.*, 1995). La valeur de C^{J}_{Ei} est donc intimement liée à l'état métabolique dans lequel ce coefficient est déterminé.

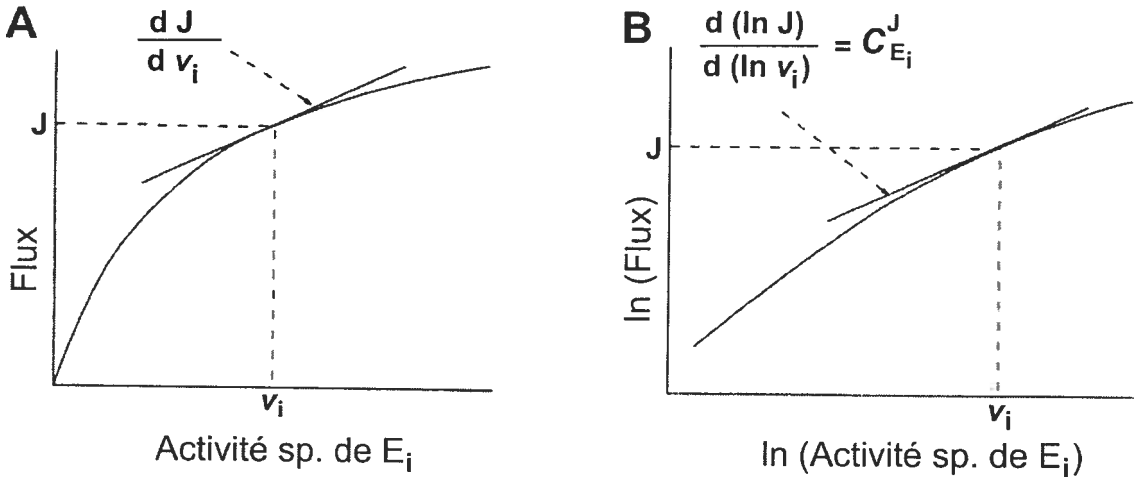

Figure 1.2 : Détermination graphique du coefficient de contrôle de flux C^{J}_{Ei} de l'enzyme E_i sur le flux J (inspiré de Fell, 1997).

A) Le coefficient C^{J}_{Ei} de l'enzyme E_i d'activité spécifique v_i sur le flux J est la pente de la tangente à la courbe dJ/dv_i multipliée par le facteur v_i/J. B) Dans une représentation logarithmique, C^{J}_{Ei} est la pente de la tangente à la courbe. L'intérêt de cette représentation est que la valeur de C^{J}_{Ei} est indépendante des unités de mesure de J et v_i.

D'après ce qui précède, C^{J}_{Ei} est une propriété du système dans un état métabolique donné, et peut être déterminé sans que ne soient connues les propriétés cinétiques de E_i. Néanmoins, le changement de flux impulsé par E_i ne survient qu'en cas de variation de son activité v_i qui, elle, est dictée par les propriétés cinétiques de E_i et par les niveaux en substrats, produits et effecteurs propres à cet état métabolique (Fell, 1997). C^{J}_{Ei} est donc la résultante des propriétés du système métabolique à l'étape de E_i et des propriétés intrinsèques de E_i, même si ces dernières n'apparaissent pas dans le calcul du coefficient (Kacser *et al.*, 1995). L'effet de chaque métabolite substrat, produit ou effecteur de E_i sur son activité v_i est quantifié par le coefficient d'élasticité de E_i relatif à ce métabolite, dans un état métabolique donné (Kacser *et al.*, 1995). Le lien direct entre C^{J}_{Ei} et les propriétés cinétiques de E_i est alors mis en évidence en exprimant C^{J}_{Ei} en fonction des coefficients d'élasticité de E_i et d'enzymes auxquelles elle est liée par ses substrats, produits ou effecteurs (Kacser *et al.*, 1995). Par ailleurs, le FCC et le coefficient d'élasticité ont servi à plusieurs démonstrations réfutant la thèse classique d'un flux contrôlé par une seule enzyme située en début de voie et soumise à un rétrocontrôle (Kacser *et al.*, 1995; Fell, 1997). La MCA montre, notamment, que l'introduction d'une boucle de rétrocontrôle dans une voie, comme celle du PEP sur la PFK dans la glycolyse végétale, déplace le contrôle du flux sur les enzymes situées en aval de la boucle (Fell, 1997; Thomas *et al.*, 1997). L'avantage du rétrocontrôle serait plutôt de minimiser les variations des niveaux d'intermédiaires et donc, le temps et l'énergie nécessaires pour atteindre un nouvel état stationnaire lors d'un changement de flux (Fell, 1997). Il s'agit alors d'un mécanisme favorisant l'homéostasie des intermédiaires de la voie, qui concerne donc la régulation de celle-ci et non pas le contrôle de son flux. En d'autres termes, une enzyme soumise à un rétrocontrôle aurait une forte capacité régulatrice sur la voie mais une faible capacité de contrôle sur son flux, contredisant ainsi les préceptes de l'analyse classique (Fell, 1997).

Cette partie nous a donc amenés à nuancer les notions de régulation et de contrôle de flux. Les études cinétiques réalisées à ce jour ont permis d'élaborer un schéma de régulation de la glycolyse végétale dans lequel la PFK, la PEPC et la PK occupent une place centrale (Plaxton, 1996). D'après ces mêmes études, l'ATP ne semble pas jouer un rôle aussi important dans la régulation glycolytique des plantes que dans celle des animaux

(Plaxton, 1996). Par contre, les cycles du Suc et du Glc/Glc-P émergent comme un mécanisme de contrôle du flux glycolytique par l'ATP, dont il semble exister des variantes chez divers Eucaryotes et Procaryotes (Thomas et Fell, 1998; Koebmann *et al.*, 2002; Urbanczyk-Wochniak *et al.*, 2003; Alonso *et al.*, 2005). Les études quantitatives sur le contrôle du flux glycolytique sont rares chez les végétaux, et ont longtemps consisté en une quête vaine d'enzymes limitantes selon les concepts de l'analyse classique (Fell, 1997). Une avancée majeure a été de montrer par la MCA et par des expériences de transgénèse qu'il est faux d'attribuer ainsi le monopole du contrôle de flux aux enzymes régulatrices de la voie (Fell, 1997; Thomas *et al.*, 1997). Au contraire, la MCA établit que le contrôle du flux glycolytique est réparti sur toutes les enzymes de la voie, la contribution de chacune étant quantifiée par son FCC (Heinrich et Rapoport, 1974; Kacser *et al.*, 1995). La mesure des FCCs des enzymes de la glycolyse promet donc une avancée considérable dans notre compréhension du contrôle de son flux. Ceci n'exclut pas de recourir aux méthodes éprouvées de l'approche dite "réductionniste" pour étudier les propriétés et la régulation de chaque enzyme isolée, du gène à l'activité (Fell, 1997). Ainsi, la caractérisation cinétique et moléculaire des enzymes glycolytiques demeure un complément indispensable aux études *in vivo* des mécanismes de régulation glycolytique et de contrôle de son flux. Néanmoins, devant l'ampleur d'une telle tâche, quelle enzyme glycolytique étudier en priorité ? La partie suivante énumère les raisons d'explorer le rôle de l'hexokinase (HK) dans la régulation de la glycolyse végétale et le contrôle de son flux.

1.3 Les raisons d'étudier l'importance de l'hexokinase dans la régulation de la glycolyse végétale et le contrôle de son flux

1.3.1 L'importance suggérée, mais non caractérisée, de l'HK dans le métabolisme primaire végétal

Face au manque de données sur les enzymes glycolytiques végétales, notre choix d'investiguer l'HK a été influencé par des données sur des HKs de mammifères aux FCCs exceptionnellement élevés parmi les études de MCA recensées (Fell, 1997). Ainsi, le FCC de l'HK sur la glycolyse, ou sa partie supérieure, atteignait 0,7–1 dans des érythrocytes, des

cellules de foie, de cœur, de pancréas, d'insulinome et de muscle de mammifères (Rapoport *et al.*, 1974; Meléndez-Hevia *et al.*, 1992; Kashiwaya *et al.*, 1994; Sweet et Matschinsky, 1995; Wang et Iynedjian, 1997a; Puigjaner *et al.*, 1997). Ces travaux ont donc démontré que l'HK exerce un fort contrôle sur le flux glycolytique dans plusieurs organes de mammifères. Chez les plantes, les études de MCA sur le contrôle du flux glycolytique font défaut, et les quelques valeurs de FCCs publiées ne concernent pas l'HK (Thomas *et al.*, 1997). Néanmoins, des expériences de manipulation génétique menées chez la tomate (*Solanum lycopersicum*) ou la pomme de terre ont mis en évidence l'importance de l'HK dans le métabolisme primaire végétal. Ainsi, des tissus photosynthétiques de tomate surexprimant une HK d'*Arabidopsis thaliana* avaient une activité photosynthétique réduite et entraient rapidement en sénescence (Dai *et al.*, 1999). Les feuilles et fruits transgéniques comportaient des niveaux d'acides aminés plus élevés que les organes contrôles (Roessner-Tunali *et al.*, 2003). Par contre, leur croissance, leurs niveaux en Glc et Fru, et leurs ratios ATP/ADP étaient fortement réduits par rapport aux contrôles et ce, tout au long du développement dans le cas des fruits (Dai *et al.*, 1999; Roessner-Tunali *et al.*, 2003; Menu *et al.*, 2004). La modification d'activité HK dans des tubercules de pomme de terre n'a pas abouti à de tels changements dans leurs masses fraîches et leurs niveaux d'amidon, Suc, Fru, Glc et hexoses-Ps (Veramendi *et al.*, 1999; Veramendi *et al.*, 2002). En revanche, les feuilles des sousexpresseurs d'HK accumulaient de l'amidon et du Glc durant la nuit, désignant donc l'HK comme une étape obligatoire du catabolisme de l'amidon chloroplastique chez la pomme de terre (Veramendi *et al.*, 1999). L'HK a également été impliquée dans le métabolisme du stress. Des niveaux insuffisants d'activité HK ont été identifiés comme la cause principale du déclin du flux glycolytique et du taux de survie de racines de maïs en anoxie (Bouny et Saglio, 1996). L'acclimatation de ces racines a permis le maintien du flux glycolytique en anoxie, lequel a été attribué à une hausse de l'activité HK et à une baisse de son inhibition (Bouny et Saglio, 1996). Ces résultats suggèrent donc un fort contrôle de l'HK sur le flux glycolytique dans les racines de maïs en anaérobiose (Bouny et Saglio, 1996).

Les travaux présentés ont donc révélé l'importance de l'HK dans le métabolisme de plusieurs organes végétaux. L'approche transgénique semble adéquate à une étude

complémentaire du rôle de l'HK dans la régulation glycolytique, et à une mesure de son FCC sur le flux glycolytique. Cependant, la modulation des niveaux de protéines d'HK par transgénèse en vue d'étudier son rôle dans le métabolisme, peut affecter deux aspects majeurs de sa biochimie qu'il importe maintenant de considérer. Il s'agit de l'implication de l'HK dans une fonction indépendante de son activité phosphorylante, et de son existence sous plusieurs isoformes dans la plupart des tissus étudiés.

1.3.2 L'HK remplit deux fonctions distinctes chez les plantes

Dans le règne végétal, l'HK exerce non seulement une fonction catalytique en tant qu'enzyme de la glycolyse, mais aussi une fonction distincte, non-catalytique, dans la perception des hexoses (Frömmer *et al.*, 2003; Harrington et Bush, 2003). Les plantes utilisent les sucres comme des nutriments et des composés structuraux, mais aussi comme des signaux qui régissent leur métabolisme, et leur croissance et développement (Rolland *et al.*, 2006). Au niveau moléculaire, les sucres régulent l'expression de gènes impliqués dans la régulation du cycle cellulaire, la photosynthèse, le métabolisme carboné et azoté, la réponse aux stress, la germination, la reproduction, ou encore la sénescence (Koch, 1996; Rolland *et al.*, 2002). L'approche transgénique a permis de démontrer que l'HK était impliquée dans la perception des hexoses. Des plantules d'*Arabidopsis* surexprimant une HK de cette même espèce étaient hypersensibles tandis que les sousexpresseurs étaient hyposensibles aux effets du Glc sur leur croissance et sur l'expression de gènes photosynthétiques (Jang *et al.*, 1997). Les effets sur la croissance, l'activité photosynthétique et la sénescence de tissus chlorophylliens de tomate surexprimant cette HK ont également été attribués à sa fonction de senseur d'hexoses (Dai *et al.*, 1999). Ces derniers auteurs ont toutefois reconnu que leurs résultats ne pouvaient être interprétés en termes de cette seule fonction, ne pouvant exclure la possibilité que des changements dans le métabolisme en aval de l'HK aient participé aux effets observés. Cette critique a été émise à l'encontre des différentes études focalisant sur la fonction de senseur d'hexoses alors que l'activité HK avait aussi été modifiée par transgénèse (Halford *et al.*, 1999). La démonstration claire et définitive que les fonctions catalytique et de senseur d'hexoses de l'HK sont distinctes et indépendantes, aura nécessité la caractérisation de mutants *gin2*

d'*Arabidopsis* insensibles au Glc. L'introduction dans ces mutants de protéines recombinantes d'HK dépourvues uniquement de leur activité phosphorylante, a permis de restaurer la fonction de perception d'hexoses chez ces transformants, d'après des tests d'expression de gènes régulés par le Glc (Moore *et al.*, 2003). Preuve fut donc faite que la fonction de l'HK en tant que senseur d'hexoses ne requérait ni l'activité phosphorylante de l'HK, ni aucun intermédiaire issu du métabolisme du Glc initié par la réaction de l'HK (Moore *et al.*, 2003).

La critique de Halford *et al.* (1999) n'en reste pas moins pertinente : le recours à la transgénèse pour étudier la fonction de senseur d'hexoses de l'HK présente le défaut de perturber aussi sa fonction catalytique. Il est donc nécessaire d'interpréter avec précaution les phénotypes de sur- et sousexpresseurs d'HK sans omettre l'une ou l'autre des fonctions de l'HK. À titre d'exemple, Veramendi *et al.* (1999) ont montré que l'HK jouait un rôle majeur dans le catabolisme de l'amidon chloroplastique dans les feuilles de pomme de terre. Toutefois, l'expression de cette HK de pomme de terre dans les sousexpresseurs d'HK de tomate a permis de recouvrer la fonction de senseur d'hexoses (Veramendi *et al.*, 2002). Ces résultats suggèrent qu'une même HK peut exercer une fonction ou l'autre selon le contexte métabolique, qui peut varier avec l'espèce, l'organe ou le stade de développement. Les mécanismes régissant l'implication de l'HK dans l'une ou l'autre fonction resteraient alors à définir. La question est soulevée, également, de savoir si une même HK peut assurer les deux fonctions simultanément dans la cellule. Il ressort que toute étude du rôle de l'HK dans la régulation glycolytique et de son niveau de contrôle sur le flux glycolytique nécessite de considérer si la fonction de senseur d'hexoses a été modifiée.

1.3.3 Les organes et tissus végétaux expriment plusieurs isoformes HK

L'HK est présente dans les organes et tissus végétaux sous plusieurs isoformes qui se distinguent, entre autres, par leurs localisations subcellulaires. Ainsi, dans les racines de maïs, les fractions issues du cytosol, de la membrane mitochondriale et du Golgi contenaient des isoformes HK actives (da-Silva *et al.*, 2001). Chez la tomate, trois isoformes ont été localisées dans la membrane mitochondriale, et une dans le stroma

(Kandel-Kfir *et al.*, 2006; Damari-Weissler *et al.*, 2006). Dans les feuilles d'épinard, une immunodétection a permis de distinguer une isoforme HK cytosolique soluble, et une autre ancrée dans la membrane externe de l'enveloppe chloroplastique (Wiese *et al.*, 1999). Enfin, une HK a été localisée dans les noyaux de protoplastes d'*Arabidopsis* (Yanagisawa *et al.*, 2003). Ces observations soulèvent la question de la signification physiologique de multiples isoformes HK dans les cellules végétales, de la redondance de leurs rôles ou d'une éventuelle spécialisation. À cet égard, plusieurs auteurs ont émis l'hypothèse que leurs propriétés cinétiques et leurs localisations subcellulaires sont des adaptations à des rôles spécifiques et individuels (Wiese *et al.*, 1999; da-Silva *et al.*, 2001; Olsson *et al.*, 2003; Cho *et al.*, 2006a). Ainsi, les variations du métabolisme carboné au cours du développement du tubercule de pomme de terre ont été corrélées aux variations d'activité HK dues aux sensibilités contrastées des isoformes HK aux variations de pH et de Glc (Renz et Stitt, 1993). Dans les racines de maïs, l'isoforme non-cytosolique était inhibée par l'ADP et par des inhibiteurs de la perception des hexoses, tandis que l'isoforme cytosolique y était insensible (Galina et da-Silva, 2000; da-Silva *et al.*, 2001). Ces auteurs ont donc proposé que l'isoforme HK non-cytosolique est un senseur d'hexoses et de l'état énergétique, et que l'isoforme cytosolique est consacrée à la fonction catalytique dans la glycolyse. Néanmoins, cette hypothèse n'a pas été vérifiée expérimentalement. Enfin, chez *Arabidopsis* les isoformes AtHXK1 et AtHXK2 ont été localisées sur la face cytosolique de la membrane externe mitochondriale, AtHXK1 pouvant se trouver aussi dans le noyau (Giegé *et al.*, 2003; Cho *et al.*, 2006b). AtHXK1 et AtHXK2 ont été impliquées dans la fonction de perception des hexoses (Jang *et al.*, 1997; Moore *et al.*, 2003). En particulier, il a été montré qu'AtHXK1 interagit avec des partenaires de signalisation dans le noyau, et que ces interactions sont à la base des effets spécifiques du Glc sur la transcription de gènes photosynthétiques (Cho *et al.*, 2006b). Cependant, le génome d'*Arabidopsis* comporte quatre autres séquences d'HK qui n'ont pas été caractérisées (Rolland *et al.*, 2006). Il n'y a donc pas d'espèce végétale pour laquelle les rôles physiologiques de toutes les isoformes HK ont été clairement établis.

Les données présentées ci-dessus reflètent la difficulté d'établir la signification physiologique de multiples isoformes HK dans les cellules végétales. Les progrès notables

ont été de prouver l'implication d'une isoforme dans la fonction catalytique ou de perception des hexoses, ou de caractériser ou localiser les isoformes présentes dans la cellule sans toutefois élucider leurs rôles respectifs. Ces résultats fragmentaires montrent la nécessité d'intégrer les données de biochimie, biologie moléculaire et génétique sur l'HK dans un schéma global propre à clarifier les rôles des isoformes et leurs contributions aux deux fonctions de l'HK. Pour les mêmes raisons, il importe de poursuivre l'effort de caractérisation d'HKs végétales purifiées. Ces différents besoins nous ont menés à formuler trois objectifs principaux pour la présente thèse, qui ont abouti à deux articles publiés et un soumis à publication.

1.4 Objectifs

Au début de ce projet de doctorat, plusieurs aspects de la signification physiologique de l'HK étaient mal compris malgré les démonstrations de son importance dans le métabolisme végétal. L'une des raisons à cela tenait aux difficultés d'étudier cette enzyme à cause de sa faible abondance dans les tissus végétaux. De plus, son existence sous plusieurs isoformes était établie pour divers organes et tissus, mais sans connaître leurs abondances relatives ni les rôles qui leur étaient associés. Enfin, il subsistait une controverse sur le fait que les fonctions de l'HK dans le métabolisme et la perception des hexoses soient ou non distinctes et indépendantes. Dans un tel contexte, nous avons décidé d'organiser mon projet de recherche selon les trois objectifs décrits ci-dessous.

1.4.1 Revue de littérature sur la signification physiologique de multiples isoformes HK chez les plantes

Lorsque j'ai commencé mon doctorat, il était clairement établi que les plantes expriment plusieurs gènes HK indépendants, comme c'est le cas chez les animaux. Plusieurs études avaient également mis en évidence l'existence d'isoformes HK dans divers tissus végétaux (Turner *et al.*, 1977; Miernyk et Dennis, 1983; Doehlert, 1989; Schnarrenberger, 1990; Renz *et al.*, 1993; Guglielminetti *et al.*, 2000). Par contre, aucune

information n'était disponible quant aux patrons d'expression des gènes HK végétaux. Des recherches préliminaires dans des bases de données en ligne nous ont permis d'identifier 6 gènes HK exprimés chez la plante modèle *A. thaliana*. Nous avons utilisé les ressources bioinformatiques disponibles afin d'étudier l'expression de ces gènes et répondre aux deux questions suivantes :

i) Les gènes HK sont-ils co-régulés dans les différents organes d'*A. thaliana*, ou existe-t-il un patron d'expression spécifique à chacun ?

ii) Ces gènes HK sont-ils régulés par des changements des conditions environnementales ?

Les données bioinformatiques obtenues ont été compilées dans une revue de la littérature sur la signification physiologique d'une pluralité d'isoformes HK chez les plantes. Nous avons examiné la possibilité que leurs patrons d'expression, propriétés biochimiques et localisations subcellulaires servent des rôles individuels en lien avec les fonctions de l'HK dans le métabolisme et la perception des hexoses. Cette revue a été publiée dans *Phytochemistry*, et constitue le chapitre 2 de la présente thèse.

1.4.2 Caractérisation moléculaire et cinétique d'une HK recombinante végétale purifiée

L'activité HK a été découverte dans un extrait de levure par Meyerhof il y a 80 ans (Meyerhof, 1927). La première HK végétale a été décrite par Saltman (1953). Il est toutefois remarquable qu'au début de ce projet, aucune HK végétale n'avait encore été purifiée à homogénéité, que ce soit sous forme native à partir d'un tissu végétal ou sous la forme d'une protéine recombinante. La purification partielle de 3 isoformes HK du tubercule de pomme de terre constituait probablement la caractérisation la plus avancée d'une HK végétale (Renz *et al.*, 1993; Renz et Stitt, 1993). Le manque d'informations sur les propriétés d'HKs végétales pures contraste avec la richesse de données sur d'autres enzymes du métabolisme primaire (Plaxton, 1996), et constitue un handicap certain à notre compréhension du rôle de l'HK chez les plantes. Parmi les raisons d'une telle lacune, il faut citer les faibles niveaux d'activité HK dans les tissus étudiés : par exemple, des activités

spécifiques de 0,01 U/mg protéine ont été rapportées dans le tubercule de pomme de terre (Renz *et al.*, 1993). D'autre part, de multiples isoformes HK coexistent dans tous les tissus végétaux investigués, ce qui multiplie les étapes nécessaires à leur purification. Enfin, des travaux récents dans notre laboratoire ont montré que certaines isoformes HK sont très stables alors que d'autres sont sujettes à une protéolyse durant leur extraction du tubercule de pomme de terre (M.-C. Moisan et J. Rivoal, données non publiées). Cette instabilité rend leur purification délicate puisqu'elle peut aboutir à des isoformes aux propriétés cinétiques altérées.

Nous avons pallié les difficultés d'une purification "classique" à partir de tissus végétaux en utilisant *E. coli* comme système hétérologue pour l'expression et la purification d'une HK recombinante. Nous avons ainsi pu déterminer les propriétés cinétiques de l'isoforme ScHK2 de *S. chacoense*, dont l'ADNc a servi aussi au troisième objectif de recherche. Ces travaux constituent la première caractérisation cinétique d'une HK recombinante végétale purifiée jusqu'à homogénéité électrophorétique. Les difficultés techniques rencontrées lors de l'expression de ScHK2 dans *E. coli* peuvent expliquer qu'il y ait eu peu de caractérisations d'HKs recombinantes végétales publiées auparavant. Ces difficultés nous ont amenés à étudier la séquence primaire de ScHK2, et à attribuer à son extrémité N-terminale un rôle inédit de ciblage vers la membrane plasmique chez les plantes. Nous avons également assigné à ScHK2 un rôle lié à la fonction catalytique de l'HK, sur la base de ses propriétés cinétiques et de sa séquence N-terminale. Ces travaux ont fait l'objet d'une publication dans la revue *Protein Expression and Purification*, et sont décrits dans le chapitre 3 de cette thèse.

1.4.3 Approche transgénique dans l'étude de la fonction catalytique de l'HK

L'HK engage les hexoses dans la glycolyse en catalysant leur phosphorylation sur le sixième carbone selon une réaction irréversible et ATP-dépendante. Sa position en amont de la voie et sa spécificité relativement large vis-à-vis des hexoses font de l'HK un site potentiel de contrôle du flux glycolytique chez les Eucaryotes. Des études ont montré que chez la levure, l'HK exerce un contrôle significatif sur le flux glycolytique avec des FCCs

compris entre 0,2 et 0,5 (Aon et Cortassa, 1998). Un tel contrôle du flux par l'HK présente un danger inhérent à la structure de la glycolyse, qui évoque le fonctionnement d'un moteur à injection turbo : celui d'épuiser l'ATP en amont avant de pouvoir le régénérer en aval de la voie (Teusink *et al.*, 1998). En cas d'abondance du Glc, une hausse brutale du flux impulsée par l'HK entraîne une production massive de G6P qui peut séquestrer le P_i destiné à la production d'ATP, menant à un blocage de la glycolyse qui prive alors la cellule d'ATP (Teusink *et al.*, 1998). Toutefois, il a été démontré que du tréhalose-6-phosphate (T6P) formé à partir du G6P inhibe l'activité HK de levure, et empêche une hausse excessive du flux glycolytique en cas d'abondance du Glc. Le fort contrôle de l'HK sur la glycolyse va donc de pair avec un système de régulation évitant le blocage de la voie et l'épuisement de l'ATP. Chez les mammifères, l'HK contrôle fortement le flux glycolytique dans plusieurs types cellulaires, avec des FCCs supérieurs à 0,7 (Rapoport *et al.*, 1974; Meléndez-Hevia *et al.*, 1992; Kashiwaya *et al.*, 1994; Sweet et Matschinsky, 1995; Wang et Iynedjian, 1997a; Puigjaner *et al.*, 1997). D'autres mécanismes propres aux cellules animales semblent intervenir de sorte que le contrôle élevé de l'HK ne puisse provoquer le blocage de la glycolyse par une hausse excessive de son flux. Citons notamment l'inhibition de l'activité HK par le G6P ou par la GKRP, la protéine régulatrice de la "glucokinase" (HK IV) (Iynedjian, 1998). Chez les plantes, le contrôle exercé par l'HK sur le flux glycolytique n'a jamais été quantifié, fait regrettable car les modèles de la levure et des animaux ne peuvent être aisément transposés aux plantes. Ceci est dû entre autres au fait que la glycolyse végétale diffère nettement de la voie animale ou de celle des microorganismes dans sa structure et sa régulation (Plaxton, 1996). Ainsi, la glycolyse végétale se distingue par sa compartimentation, sa structure en réseau de réactions redondantes, et son alimentation principalement à partir du Suc. D'autre part, si le T6P a récemment émergé comme une molécule régulatrice du métabolisme primaire des plantes, il n'agit pas en tant qu'inhibiteur de l'HK comme chez la levure (Paul, 2007).

Face au manque de données chez les plantes, nous avons entrepris de quantifier expérimentalement le contrôle de l'HK sur le flux glycolytique. L'ADNc de ScHK2 de *S. chacoense* nous a servi à sur- et sousexprimer l'HK dans des racines de pomme de terre. La grande homologie de séquence entre ScHK2 de *S. chacoense* et les isoformes StHK1 et

StHK2 de *S. tuberosum* rendait alors très probable le succès de la stratégie antisens. Par ailleurs, les plants de pomme de terre ont été transformés avec *Agrobacterium rhizogenes*, ce qui a rapidement généré des clones racinaires issus chacun d'une seule cellule transformée. Les racines transgéniques constituent un matériel clonal facile à sous-cultiver et multiplier, et qui peut servir à régénérer des plantes entières. De plus, l'utilisation de ce matériel expérimental non-photosynthétique permet d'isoler la glycolyse d'un éventuel apport en sucres produits par photosynthèse. Ceci simplifie l'interprétation des résultats de marquages avec des substrats de la glycolyse radioactifs utilisés comme traceurs. Nous avons donc généré et caractérisé une population de clones racinaires comportant des niveaux différents d'activité HK, afin de répondre aux questions suivantes :

i) Le FCC de l'HK sur le flux glycolytique est-il aussi élevé chez les plantes que chez la levure ou les mammifères ?

ii) Dans le cas d'un fort contrôle de l'HK sur l'étape de phosphorylation des hexoses, existe-t-il un mécanisme empêchant un blocage de la glycolyse par séquestration du P_i dans les hexoses-Ps ?

iii) La manipulation de l'activité HK dans les tissus hétérotrophes a-t-elle des effets pléiotropiques dans d'autres zones du métabolisme ?

Nous avons eu recours au marquage de racines transgéniques au 2-désoxy-D-[U-^{14}C]glucose ([U-^{14}C]DOG) pour traiter les deux premières questions. Le DOG, utilisé dans diverses études d'HKs animales et végétales, nous a permis ici de mesurer le flux glycolytique *in vivo*, et d'en déduire le FCC de l'HK sur sa propre étape de phosphorylation des hexoses. Nous avons aussi mis en évidence une métabolisation du 2-désoxy-D-[U-^{14}C]glucose-6-phosphate ([U-^{14}C]DOG6P) survenant à des taux proportionnels aux niveaux d'activité HK dans les clones racinaires. La troisième question a été abordée d'une part en mesurant la croissance des racines transformées, d'autre part en dosant un large spectre de métabolites tels que des sucres, des acides aminés et organiques, et des hexoses-Ps. Sur la base de ces résultats, nous avons montré le rôle clé de l'HK dans la régulation de la croissance racinaire, et avons prouvé que l'HK contrôle fortement l'entrée des hexoses dans la glycolyse végétale. Nous avons pu également présenter et étayer une hypothèse sur l'implication de l'HK, ainsi que des cycles du Suc et du Glc/Glc-P, dans la régulation de la

glycolyse. L'ensemble de ces données est regroupé dans le chapitre 4 de cette thèse, sous forme d'un article soumis à la revue *Journal of Biological Chemistry*.

Chapitre 2.

Isozymes de l'Hexokinase Végétale : Occurrence, Propriétés et Fonctions

Claeyssen et Rivoal, 2007. Phytochem. 68, 709-731.

Contribution des coauteurs :

Je suis l'auteur des figures et tableaux ainsi que de la quasi-totalité du texte de cette revue bibliographique.

2.1 Title page

Isozymes of plant hexokinase: Occurrence, properties and functions

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Submitted to Phytochemistry 21 July 2006.
Accepted in revised form 26 November 2006.
Published online 17 January 2007.
Claeyssen and Rivoal, 2007. Phytochem. 68, 709-731.

2.2 Preliminary material

2.2.1 Authors' profiles

Éric Claeyssen is a Ph.D. student in plant metabolic biochemistry at the Institut de Recherche en Biologie Végétale of the Université de Montréal. He received a M.Sc. degree from the University of Manitoba with Dr. Robert Hill. His research focuses on the function of plant hexokinase in nonphotosynthetic tissues.

Jean Rivoal is an Assistant Professor at the Institut de Recherche en Biologie Végétale of the Université de Montréal. He holds a Doctoral degree in Health and Biology from the Université de Bordeaux II, France. Dr. Rivoal held post-doctoral positions with Dr. Andrew Hanson at the Université de Montréal and with Dr. David Turpin and Dr. William Plaxton at Queen's University. His research interests concern the regulation of plant cytosolic glycolysis in nonphotosynthetic tissues. Experimental approaches used in his laboratory put particular emphasis on the characterization of purified enzymes and on the study of the relationship between enzyme amounts and metabolic flux in transgenic plants.

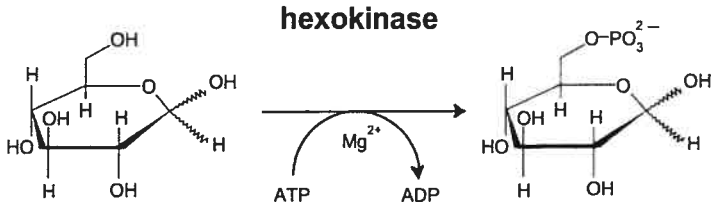
2.2.2 Keywords

Arabidopsis; *Cruciferae*; Carbon metabolism; Glucose; Glycolysis; Hexokinase; Hexose sensing; Microarray; Sequence analysis; *Solanaceae*.

2.2.3 Graphical abstract

Plant hexokinase fulfills a dual function as a glycolytic enzyme and a hexose sensor, and exists in multiple isoforms in all organs and tissues investigated. We discuss how the

subcellular localizations and biochemical properties of these hexokinase isoforms may suit them to specific roles in primary metabolism and/or hexose sensing.



2.3 Abstract

Hexokinase (HK) occurs in all phyla, as an enzyme of the glycolytic pathway. Its importance in plant metabolism has emerged with compelling evidence that its preferential substrate, glucose, is both a nutrient and a signal molecule that controls development and expression of different classes of genes. A variety of plant tissues and organs have been shown to express multiple HK isoforms with different kinetic properties and subcellular localizations. Although plant HK is known to fulfill a catalytic function and act as a glucose sensor, the physiological relevance of plural isoforms and their contribution to either function are still poorly understood. We review here the current knowledge and hypotheses on the physiological roles of plant HK isoforms that have been identified and characterized. Recent findings provide hints on how the expression patterns, biochemical properties and subcellular localizations of HK isoforms may relate to their modes of action. Special attention is devoted to kinetic, mutant and transgenic data on HKs from *Arabidopsis thaliana* and the *Solanaceae* potato, tobacco, and tomato, as well as HK gene expression data from *Arabidopsis* public DNA microarray resources. Similarities and differences to known properties of animal and yeast HKs are also discussed as they may help to gain further insight into the functional adaptations of plant HKs.

2.4 Hexokinase: An 80-year-old enzyme still full of life

HK (EC 2.7.1.1, Supplementary Table 2.1) was first described in a seminal paper by Otto Meyerhof (Nobel Prize in Physiology or Medicine in 1922) in an extract from baker's yeast (*Saccharomyces cerevisiae*) (Meyerhof, 1927). However, its reaction was elucidated more than a decade later (Von Euler and Adler, 1935; Meyerhof, 1935; Colowick and Kalckar, 1943) as summarized below:

$$\text{hexose} + \text{ATP} \rightarrow \text{hexose-6-phosphate} + \text{ADP} \qquad (2.1)$$

The elucidation of the Embden–Meyerhof–Parnas pathway in the 1940s established HK as a glycolytic enzyme in yeast and animals (Plaxton, 1996). The interest for plant HK came with evidence for the presence of a functional glycolytic pathway in plants (Stumpf, 1952; Beevers and Gibbs, 1954). The concomitant detection of the oxidative pentose-phosphate pathway (OPPP) in plants (Axelrod *et al.*, 1953) demonstrated another route for the metabolism of D-glucose-6-phosphate (G6P), a product of HK. The first isolation and characterization of a plant HK (Saltman, 1953) was therefore a significant milestone. Using wheat (*Triticum aestivum*) germ extracts, Saltman (1953) demonstrated the existence of HK in a higher plant, which had only been inferred until then (Saltman, 1953). Early studies on plant HKs were also instrumental in providing evidence for soluble and particulate forms of the enzyme (Millerd *et al.*, 1951; Saltman, 1953). Recent developments have now provided strong experimental evidence that HK activity is associated with the mitochondrion and plastids (Giegé *et al.*, 2003; Wiese *et al.*, 1999). The interest in this 80-year-old enzyme has notably been renewed with the discovery that HK fulfills catalytic and sensing functions in plants (Moore *et al.*, 2003).

It is now well established that HK is present in virtually all living organisms as part of the ancillary glycolytic pathway (Cárdenas *et al.*, 1998). In plants, the enzyme phosphorylates several hexoses including D-glucose (Glc), D-fructose (Fru), D-mannose (Man) and D-galactose (Gal). Plant HK is thus distinct from glucokinase (GK), fructokinase (FK), mannokinase (MK) and galactokinase (GalK) that can be highly specific to Glc, Fru, Man and Gal, respectively (Cárdenas *et al.*, 1998). Moreover, available FK and GalK protein sequences share no obvious homology with those of HK (Dai *et al.*, 2002). As for

MK and GK, no molecular or biochemical evidence of their existence in plants has been provided yet. The so-called GKs isolated to date could phosphorylate Fru or Man in addition to Glc, although with sometimes extremely low efficiency (Turner *et al.*, 1977; Doehlert, 1989; Martinez-Barajas and Randall, 1998). Therefore, it has been concluded that these GKs in fact belong to the HK family, and that no true GKs have been found yet in Eukaryotes (Cárdenas *et al.*, 1998; Dai *et al.*, 2002).

HK's specificity for several hexose substrates makes this enzyme a gateway to glycolysis for hexoses arising from D-sucrose (Suc) or transitory starch degradation. Moreover, the main product of HK, G6P, not only feeds cytosolic and plastidic glycolysis but is also a precursor for the OPPP and for fatty acid, starch and cell wall polysaccharide biosyntheses in their respective cell compartments (Neuhaus and Emes, 2000; Seifert, 2004). In addition to its catalytic role, HK acts as a hexose sensor and mediates hexose responses in gene expression, germination, growth, vegetative and reproductive development, stress and senescence (Rolland *et al.*, 2002). Targeted mutagenesis and HK mutants have been used to demonstrate that the Glc sensing function of HK is distinct and independent from its catalytic activity (Moore *et al.*, 2003). The sensing and signaling function of plant HK has recently been reviewed as part of the signaling network that integrates environmental cues with those from sugars, hormones, nutrients and stresses to govern plant developmental programs (Rolland *et al.*, 2006).

Despite the cardinal importance of HK in plant life, several aspects of its physiology and biochemistry are still obscure. In particular, HK has been observed in multiple isoforms in a wide variety of plant species and tissues (Renz *et al.*, 1993; da-Silva *et al.*, 2001; Cho *et al.*, 2006). However, the physiological significance of this heterogeneity requires clarification. The aim of this review is to explore how subcellular localizations, kinetic and regulatory properties as well as gene expression patterns of plant HK isoforms may interrelate in their individual roles and modes of action. We also present the current knowledge on the catalytic function and implication of HK in hexose sensing and signaling. Research on yeast and animal HKs is also considered as it may provide a useful basis for comparison. By integrating physiological, biochemical and molecular data, this

comprehensive review highlights the complexity of the roles and modes of action of plant HKs.

2.5 Evidence for multiple HK isoforms with different biochemical properties and implications in primary metabolism

2.5.1 A variety of metabolic pathways feed the different HK isoforms

2.5.1.1 Suc catabolism

Due to its broad specificity to hexoses, HK uses substrates from multiple metabolic pathways including Suc catabolism (Fig. 2.1). In most plants, Suc is synthesized in source leaves and transported via the phloem to sink tissues, where it is stored or catabolized for starch synthesis or for heterotrophic metabolism and growth (Quick and Schaffer, 1996). Apoplastic unloading of Suc into sink cells is mediated by Suc transporters (SUTs). Alternatively, Suc may be hydrolyzed by invertase (INV) into Glc and Fru, which are subsequently taken up by monosaccharide transporters (MSTs) (Fig. 2.1) (Lalonde *et al.*, 2004). There is convincing evidence also for endocytosis of apoplastic Suc and Glc to the vacuole (Etxeberria *et al.*, 2005a; Etxeberria *et al.*, 2005b). Within sink cells, Suc may be cleaved by INV and/or by Suc synthase (SuSy). INV exists as a vacuolar (vINV), a cytosolic (cINV) and a cell-wall bound (cwINV) isoforms (Roitsch and Gonzalez, 2004). INV activity is generally due mainly to cwINV and vINV whereas cINV activity is comparatively very low (Kim *et al.*, 2000; Thévenot *et al.*, 2005). SuSy is found in the cytosol, where it may be soluble, or attached to the actin cytoskeleton, or associated with the plasma membrane, the tonoplast or the Golgi membrane (Koch, 2004). This enzyme is also found within the mitochondrion where it possibly fulfills a non-catalytic function in signaling (Subbaiah *et al.*, 2006). SuSy catalyses the conversion of Suc and UDP into Fru and UDP-D-glucose (UDP-Glc) according to a reversible reaction, but may function with other nucleoside-5'-diphosphates (NDPs) such as ADP (Baroja-Fernandez *et al.*, 2003). Interestingly, INV and SuSy activities may mediate transitions through the prominent stages of sink organ development (Sturm and Tang, 1999; Koch, 2004; Roitsch and

Gonzalez, 2004). It appears from observations made on root, tuber, fruit, and seed that Suc degradation by vINV and cwINV predominate at sink initiation and expansion. At this stage, cwINV activity and the resultant high hexose/Suc ratio are generally associated with high cell division rates (Weber *et al.*, 1997; Koch, 2004). In the seed, for instance, evidence is based on mutant studies and histological mapping of hexose and Suc gradients (Weber *et al.*, 2005). High cwINV activity also correlates with elevated expression levels of MSTs (Sherson *et al.*, 2003; Weschke *et al.*, 2003). Transition to the later storage and maturation phases is characterized by a switch from the INV- to the SuSy path of Suc cleavage (Weber *et al.*, 1997; Sturm and Tang, 1999; Nguyen-Quoc and Foyer, 2001; Fernie *et al.*, 2002b). The rise in SuSy activity coincides with a shift from cell division to cell differentiation and elongation, possibly due to a gradual decrease of the hexose/Suc ratio (Weber *et al.*, 1997; Koch, 2004). These data suggest that the developmental stage of the organ may be of prime importance when considering the contribution of INV and SuSy activities to the supply of Glc and/or Fru to HK.

2.5.1.2 Competition for Fru between HK and FK

The Fru produced by INV and/or SuSy activities may serve as substrate for HK or, alternatively, for FK (Fig. 2.1). Numerous plant sources have been shown to express between one and three FK isoforms (Pego and Smeekens, 2000). Study of potato (*Solanum tuberosum*) tuber early development has shown high cwINV and HK activities in the mitotically active, subapical region of the non-tuberizing stolon (Appeldoorn *et al.*, 2002). Later, during tuberization, most hexose-phosphorylating activity was due to FK, which coincided with the predominant SuSy path of Suc cleavage (Renz *et al.*, 1993; Appeldoorn *et al.*, 2002; Davies *et al.*, 2005). Therefore, the relative levels of HK and FK activities correlated with a possible carbon flow via the INV and SuSy routes, respectively, at different time points in tuber development. Results from transgenic experiments have also supported the view that Fru produced by SuSy during tuberization may not feed the HK reaction for further metabolism. Tuber yield was dramatically altered in potato plants with decreased SuSy activity (Zrenner *et al.*, 1995), whereas those carrying HK antisense constructs exhibited little difference with the wild-types (Veramendi *et al.*, 1999;

Veramendi *et al.*, 2002). Transgenic tomato (*Solanum lycopersicum*) studies have shown a similar SuSy/FK combination in Suc partitioning in seed, root, stem and reproductive tissues (Odanaka *et al.*, 2002; German *et al.*, 2003). Coordinated regulation of FK and SuSy activities have been reported elsewhere (Huber and Akazawa, 1986; Hill *et al.*, 2003), suggesting a widespread occurrence of the SuSy/FK pathway of Suc degradation in plants (Pego and Smeekens, 2000; Davies *et al.*, 2005). Given the poor efficiency of HK and high efficiency of FK with Fru (Renz and Stitt, 1993; da-Silva *et al.*, 2001; Menu *et al.*, 2001; Claeyssen *et al.*, 2006), the above data suggest that Fru could be consumed almost entirely by FK in situations where the SuSy/FK and INV/HK routes may coexist.

2.5.1.3 Impact of substrate cycles on carbon flow through HK

There is evidence for substrate (futile) cycles that involve Suc synthesis and degradation. Suc synthesis occurs via SuSy, Suc-phosphate synthase (SPS) and Suc-phosphate phosphatase, whereas its degradation is mediated by SuSy (reverse reaction), cINV and vINV. These reactions are believed to govern fruit sugar content and composition (Nguyen-Quoc and Foyer, 2001). In other systems, Suc cycling has been proposed to buffer variations in metabolite concentrations or adjust metabolism to Suc supply, consuming between 5% and 80% of the ATP generated by the cell (Dieuaide-Noubhani *et al.*, 1995; Fernie *et al.*, 2002a; Rontein *et al.*, 2002). Interestingly, increasing Suc degradation activity in potato tubers by transforming plants with a yeast INV, a bacterial Suc phosphorylase or a bacterial xylose isomerase, led to increases in Suc cycling and glycolytic activity (Trethewey *et al.*, 1999; Fernie *et al.*, 2002a; Urbanczyk-Wochniak *et al.*, 2003). It was concluded that glycolytic flux may be controlled, at least in part, by processes like Suc cycling that increase the ATP demand of the cell (Fernie *et al.*, 2002a; Urbanczyk-Wochniak *et al.*, 2003). In other terms, Suc cycling may contribute to modulation of carbon flow through HK and glycolysis by way of ATP dissipation, although other unknown factors seem to be involved (Junker *et al.*, 2006).

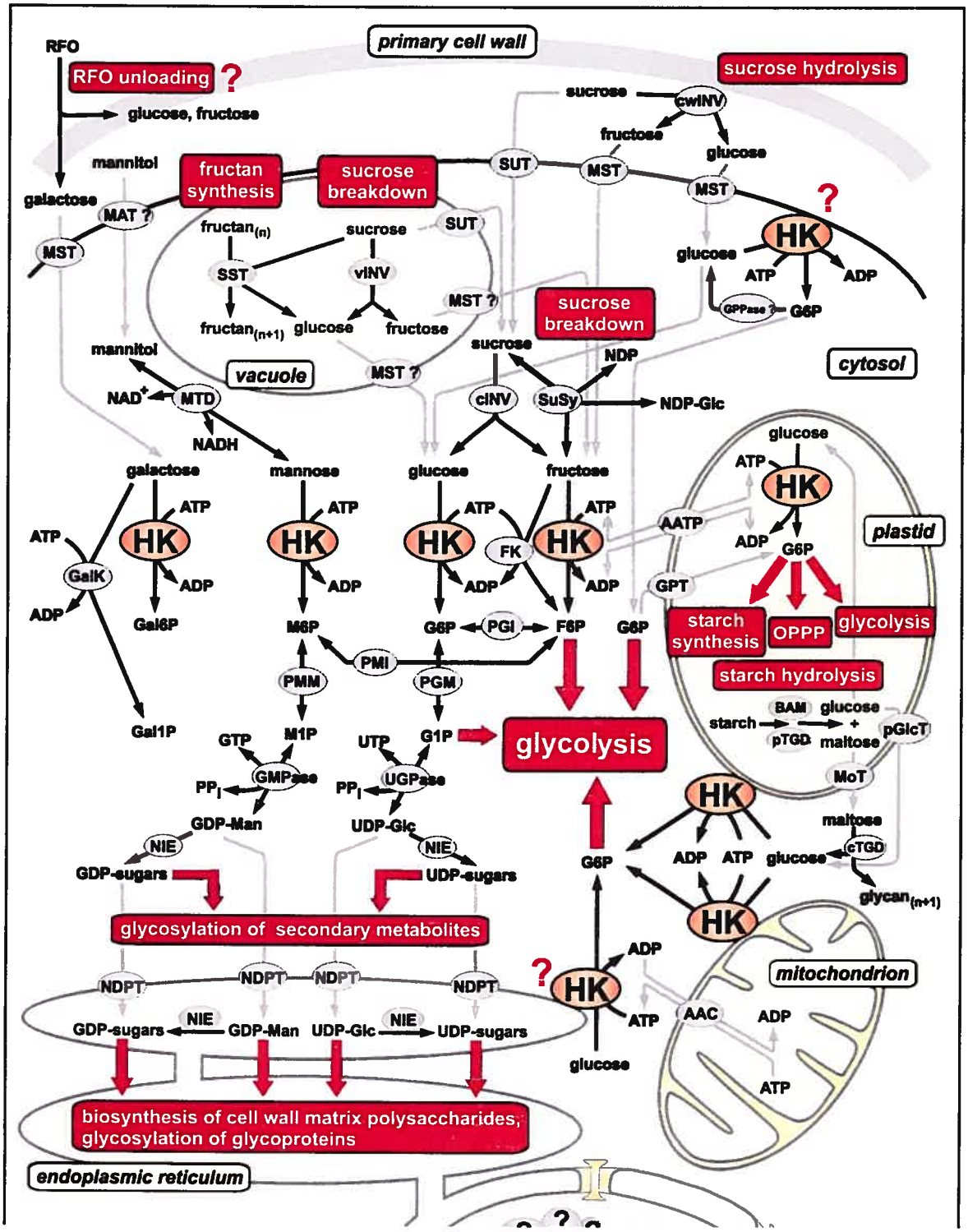

primary cell wall
RFO
RFO unloading ?
glucose, fructose
sucrose hydrolysis
sucrose
cwINV
fructose
glucose
mannitol
fructan synthesis
sucrose breakdown
SUT
MST
MST
?
galactose
MAT ?
MST
fructan(n)
sucrose
SUT
SST
vINV
MST ?
fructan(n+1)
glucose
fructose
HK
glucose
ATP
ADP
GPPase ?
G6P
sucrose breakdown
mannitol
vacuole
MST ?
sucrose
NDP
cytosol
NAD+
MTD
cINV
SuSy
NDP-Glc
NADH
glucose
ATP
galactose
mannose
glucose
fructose
HK
ATP
ADP
ATP
GalK
ADP
Gal6P
M6P
G6P
PGI
F6P
G6P
FK
AATP
GPT
plastid
PMI
PMM
PGM
starch synthesis
OPPP
glycolysis
starch hydrolysis
Gal1P
GTP
M1P
UTP
G1P
glycolysis
BAM
glucose
starch
pTGD
maltose
pGlcT
MoT
PPi
GMPase
PPi
UGPase
GDP-Man
UDP-Glc
NIE
NIE
maltose
cTGD
GDP-sugars
UDP-sugars
glycan(n+1)
glycosylation of secondary metabolites
NDPT
NDPT
NDPT
NDPT
?
ADP
mitochondrion
NIE
NIE
GDP-sugars
GDP-Man
UDP-Glc
UDP-sugars
ATP
AAC
ADP
glucose
ATP
biosynthesis of cell wall matrix polysaccharides, glycosylation of glycoproteins
endoplasmic reticulum

Figure 2.1: Schematic representation of the metabolic pathways that involve hexokinase (HK) in plant carbon metabolism.

Metabolic pathways relating to HK activity are indicated in red. Black arrows illustrate reactions while translocation of intermediates is in grey. Question marks indicate HK localization, proteins or transporters for which experimental evidence is lacking. Transport mechanisms (by facilitation or H^+-coupled cotransport) and tissue- and organelle-specificities of SUTs and MSTs (Lalonde *et al.*, 2004) are not detailed. NIE specificities are reviewed in Seifert (2004). *Metabolic pathways and intermediates:* F6P, D-fructose-6-phosphate; G1P, D-glucose-1-phosphate; G6P, D-glucose-6-phosphate; Gal1P, D-galactose-1-phosphate; Gal6P, D-galactose-6-phosphate; GDP-Man, GDP-D-mannose; M1P, D-mannose-1-phosphate; M6P, D-mannose-6-phosphate; NDP, nucleoside-5'-diphosphate; NDP-Glc, NDP-D-glucose; OPPP, oxidative pentose-phosphate pathway; PP_i, pyrophosphate; RFOs, raffinose-family oligosaccharides; UDP-Glc, UDP-D-glucose. *Enzymes and transporters:* AAC, mitochondrial ADP/ATP carrier; AATP, plastidic ATP/ADP translocator; BAM: ß-amylase, cTGD, cytosolic transglucosidase; FK, fructokinase; FTF, fructosyltransferase; GalK, galactokinase; GMPase, GDP-D-mannose pyrophosphorylase; GPPase?, putative D-glucose-phosphate phosphatase; GPT, D-glucose-6-phosphate/P_i translocator; HK, hexokinase; MAT?, putative mannitol transporter; MST, monosaccharide transporter; MST?, putative monosaccharide transporter of the tonoplast; cINV, cwINV and vINV, cytosolic, cell-wall and vacuolar isoforms of invertase, respectively; MoT, chloroplastic maltose transporter; MTD, mannitol dehydrogenase; NDPT, nucleoside-5'-diphosphate sugar transporter; NIE, nucleoside-5'-diphosphate sugar interconverting enzyme; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; pGlcT, plastidic D-glucose translocator; PMI, phosphomannose isomerase; PMM, phosphomannomutase; pTGD, plastidic transglucosidase; SuSy, D-sucrose synthase; SUT, D-sucrose transporter; UGPase, UDP-D-glucose pyrophosphorylase. EC numbers of cited enzymes are available in Supplementary Table 2.1.

An additional substrate cycle, involving Glc and Glc-P, i.e. either G6P or D-glucose-1-phosphate (G1P), has been described in maize (*Zea mays*) root tips (Fig. 2.1) (Alonso *et al.*, 2005). The high rate of Glc-P to Glc turnover was shown to consume up to 40% of the ATP generated, whereas Suc cycling used at most 6% of the ATP produced in the roots (Alonso *et al.*, 2005). Several Glc-P phosphatases have been characterized in animals, yeast or bacteria, including 2-deoxy-D-glucose-6-phosphatase, D-glucose-6-phosphatase and D-glucose-1-phosphatase (Tsujimoto *et al.*, 2000; Lee *et al.*, 2003; Mithieux *et al.*, 2004). However, sequence homology or biochemical evidence for the presence of these enzymes in plants is lacking (Claeyssen and Rivoal, unpublished observations). *In vitro* phosphatase activities with G1P and G6P have been reported, but seemed to be due to low substrate specificity of acid phosphatases (Duff *et al.*, 1989; Bozzo *et al.*, 2004; Yoneyama *et al.*, 2004). Whether these activities have physiological relevance to Glc/Glc-P cycling remains to be established. Ultimately, newly synthesized Glc may re-enter the hexose-phosphate (hexose-P) pool by way of the HK reaction, together with consumption of a new ATP molecule (Fig. 2.1). Although its role and regulation remain to be clarified, the Glc/Glc-P cycle may directly implicate HK in modulation of the energy status of the cell. This novel substrate cycle has also been ascribed the hypothetical role of raising glycolytic rate through increased energy demand, by analogy to Suc cycling (Alonso *et al.*, 2005).

2.5.1.4 Starch degradation

A major source of Glc for HK may arise with degradation of transitory starch in leaves or storage starch in seeds, roots, tubers, and fruit. In photosynthesizing leaves, starch accumulates in chloroplasts in the daytime, when photoassimilate supply exceeds the demand for Suc. This transitory starch is remobilized at night for continued synthesis and export of Suc to heterotrophic plant parts, and for the immediate metabolic requirements of the leaf cell (Preiss and Sivak, 1996). Breakdown of transitory starch has been most extensively characterized in *Arabidopsis* and potato leaves, based on mutant and transgenic studies (Zeeman *et al.*, 2004; Smith *et al.*, 2005). Attack of the starch granule in the chloroplast yields linear α-1,4-glucans that are degraded mainly by β-amylase (BAM) into maltose and, to a lesser extent, maltotriose (Fig. 2.1) (Zeeman *et al.*, 2004). Maltotriose

may be degraded into Glc by plastidic transglucosidase (pTGD) (Critchley *et al.*, 2001), and feed a stromal HK (Fig. 2.1) (Ritte and Raschke, 2003). This is very likely for chloroplasts of the moss *Physcomitrella patens*, where stromal HK accounts for 80% of total HK activity (Olsson *et al.*, 2003). In higher plants, however, stromal NtHxk2 of tobacco (*Nicotiana tabacum*) and its two candidate orthologs in *Arabidopsis*, At1g47840 and At4g37840, are expressed at low levels, if at all, in leaves (Zeeman *et al.*, 2004; Giese *et al.*, 2005). Stromal HK may rather be recruited in starch sheath chloroplasts or in non-green plastids, to feed glycolysis or the OPPP with G6P (Giese *et al.*, 2005). Alternatively, Glc may leave the chloroplast by way of a plastidic Glc translocator (pGlcT, Fig. 2.1), moving down a concentration gradient across the chloroplast envelope (Häusler *et al.*, 1998; Weber *et al.*, 2000). Indeed, stromal levels of Glc may be relatively high, e.g. 14 mM in isolated spinach (*Spinacia oleracea*) chloroplasts (Stitt and Heldt, 1981), assuming a chloroplastic volume of 25 µL/mg chlorophyll (Gerhardt *et al.*, 1987). Cytosolic Glc levels appear to be lower, e.g. 0.4 mM in potato leaves (Leidreiter *et al.*, 1995). Wiese *et al.* (1999) have suggested that an HK inserted in the chloroplastic outer envelope membrane could be particularly suited to maintain a gradient of Glc by phosphorylating Glc during export from the plastid. In accordance with a predominant β-amylolytic pathway, starch catabolites are exported to the cytosol at night almost exclusively as maltose and Glc in several species (Servaites and Geiger, 2002; Weise *et al.*, 2004). Export of maltose to the cytosol occurs via a specific maltose transporter of the chloroplast envelope (MoT, Fig. 2.1) (Niittylä *et al.*, 2004). In *Arabidopsis* leaves, maltose is then cleaved by cytosolic transglucosidase (cTGD, Fig. 2.1), which transfers one glucosyl residue onto an unknown heteroglycan acceptor and releases the second Glc (Chia *et al.*, 2004; Lu and Sharkey, 2004). Therefore, it appears that transitory starch degradation yields substantial amounts of cytosolic Glc, which may lead to a major carbon flow through the cytosolic HK reaction. Interestingly, antisense repression of cytosolic HK resulted in higher Glc and lower Suc levels in transgenic potato leaves compared to wild-types at the end of the night (Veramendi *et al.*, 1999). These data may then point to HK as an obligate step in commitment of starch catabolites to Suc synthesis and glycolysis in the leaf cell at night (Fig. 2.1).

Moreover, mutant and gene expression profiling studies suggest a common pathway of starch breakdown in other *Arabidopsis* plant organs that store it transiently (Smith *et al.*, 2005). However, this pathway is likely to differ from that occurring in storage organs. In cereal endosperm, starch breakdown takes place in nonliving tissue, in which no intra- or intercellular compartmentation remains. Linear α-1,4-glucans are probably degraded via α- and β-amylases and α-glucosidase to maltose and Glc, which can enter the embryo (Smith *et al.*, 2005). The high energy and carbon requirements for biosyntheses during germination may thus rely heavily on HK for energization of starch-derived Glc within the cereal embryo. As for starch-storing legume seeds and potato tuber, the pathway of starch breakdown has not yet been elucidated. Therefore, the degree of implication of HK is uncertain for those systems (Smith *et al.*, 2005).

2.5.1.5 Catabolism of polyols and raffinose-family oligosaccharides

A number of plants transport and store soluble sugars such as polyols (e.g. mannitol, sorbitol), raffinose-family oligosaccharides (RFOs), or a combination of those, in parallel with Suc (Turgeon, 1996). In celery, equal amounts of mannitol and Suc are translocated to sink tissues (Davis and Loescher, 1990). Mannitol is synthesized in the cytosol of leaf cells, from photosynthetic triose-phosphates via D-fructose-6-phosphate (F6P), D-mannose-6-phosphate (M6P), and D-mannitol-1-phosphate (Rumpho *et al.*, 1983; Loescher *et al.*, 1992). In sink tissues, mannitol is stored in the vacuole or oxidized directly to Man by mannitol dehydrogenase (MTD) (Stoop and Pharr, 1992). Man may be readily phosphorylated by HK into M6P, the latter entering glycolysis after isomerization to F6P by phosphomannose isomerase (PMI) (Fig. 2.1) (Fujiki *et al.*, 2001).

RFOs are of widespread occurrence in seeds, where they may serve as desiccation tolerance agents or as carbohydrate reserves for energy supply to the new seedling during germination (Keller and Pharr, 1996). Their degradation possibly involves INV, α-galactosidase and α-glucosidase (Cook *et al.*, 2004). The released Glc, Fru and Gal may subsequently feed the HK reaction or, alternatively, Gal may be phosphorylated into D-galactose-1-phosphate (Gal1P) by GalK. Accordingly, in germinating *Vicia faba* seeds, a

steady decrease in raffinose and stachyose levels was correlated to an increase in GalK activity. The latter was sufficiently high to phosphorylate all the Gal released from RFO breakdown and correspondingly, no free Gal was detected (Dey, 1983). Therefore, HK and GalK may compete for Gal during germination of legume seeds. As mentioned above, some plants use RFOs as long-distance carbon allocation forms, together with Suc (Turgeon, 1996). Eventually, their degradation in sink tissues will generate Glc, Fru and Gal and, again, HK and GalK may compete for Gal (Fig. 2.1). This may be the case for *Arabidopsis*, which has been shown to transport small amounts of raffinose (Haritatos *et al.*, 2000) and to express a GalK in all organs investigated (Kaplan *et al.*, 1997).

2.5.1.6 Fructan synthesis yields Glc

Fructans, rather than starch, are used as reserve carbohydrates by up to 15% of higher plants. Fructans are soluble Fru oligomers and polymers that are stored in the vacuole of both photosynthetic and storage cells (Vijn and Smeekens, 1999). They accumulate when photoassimilate production exceeds demand, or upon cold or drought stress (Ritsema and Smeekens, 2003). Several specific fructosyltransferases (FTFs) catalyze fructan synthesis in the vacuole, with Suc as the sole precursor (Vijn and Smeekens, 1999). Consequently, fructan accumulation releases substantial amounts of Glc, which may exit the vacuole for subsequent phosphorylation by HK (Fig. 2.1). Also, fructan degradation produces large amounts of Fru, which may serve as substrate for cytosolic HK and FK.

2.5.2 The different HKs feed various metabolic pathways

2.5.2.1 HK feeds the glycolytic pathway

HK is a glycolytic enzyme and hence, contributes to breakdown of carbohydrates to fuel respiration and provide carbon intermediates to numerous anabolic pathways (Fig. 2.1). The plant glycolytic pathway is compartmentalized (Plaxton, 1996). Therefore, the fate of glycolytic intermediates or the end product pyruvate depends on the cell compartment

where they occur. A complete, functional glycolytic pathway, present on the cytosolic face of the outer mitochondrial membrane, has been proposed to provide pyruvate directly to the mitochondrion as respiratory substrate (Giegé *et al.*, 2003). By the same token, mitochondrion-associated HK may gain preferential access to mitochondrial ATP, which is translocated to the cytosol via an ADP/ATP carrier (AAC) (Fig. 2.1) (Galina and da-Silva, 2000; Spagnoletta *et al.*, 2002). In nonphotosynthetic plastids of castor (*Ricinus communis*) oilseeds, a complete or partial glycolytic pathway may provide pyruvate to the lipid biosynthetic pathway (Neuhaus and Emes, 2000). Although compartmentalized, the cytosolic and plastidic glycolytic pathways are interconnected via highly selective metabolite transporters in the plastidic inner envelope membrane (Plaxton, 1996). Cytosolic pyruvate may thus be imported by a pyruvate carrier into the nonphotosynthetic plastid where it may serve as a precursor for fatty acid synthesis (Eastmond and Rawsthorne, 2000; Neuhaus and Emes, 2000). Similarly, G6P, phospho*enol*pyruvate (PEP) and triose-phosphates produced by cytosolic glycolysis, may enter the plastid via specific transporters to feed glycolysis and subsequent fatty acid biosynthesis (Neuhaus and Emes, 2000).

2.5.2.2 HK feeds starch biosynthesis and the OPPP

Apart from its contribution to glycolysis-derived carbon supply, HK may play a pivotal role in providing G6P to several pathways (Fig. 2.1). Thus, cytosolic G6P may enter the nonphotosynthetic plastid via a G6P/inorganic phosphate (P_i) translocator (GPT) to feed starch biosynthesis (Neuhaus and Emes, 2000). Alternatively, HK-derived G6P may feed the OPPP by way of its first enzyme, G6P dehydrogenase (G6PDH). The OPPP provides carbon for synthesis of nucleotides, aromatic amino acids and phenylpropanoids, and reductant for nitrogen assimilation and fatty acid synthesis in the plastid (Neuhaus and Emes, 2000). In rapeseed (*Brassica napus*) embryo, for example, the OPPP may produce up to 22% of the reductant needed for plastidic fatty acid synthesis (Schwender *et al.*, 2003). At least part of the OPPP occurs in both the cytosol and the (non)green plastid, as evidenced by coexistent cytosolic, plastidic and chloroplastic G6PDH activities (Kruger and von Schaewen, 2003). Therefore, both cytosolic and stromal HKs may feed G6P to the OPPP and hence, contribute to OPPP-mediated supply of carbon and reductant for plastid

metabolism. Several of the plastidic processes cited above rely heavily on ATP supply (Neuhaus and Emes, 2000). In chloroplasts, ATP is produced by photosynthesis. In nonphotosynthetic plastids, a partial glycolytic pathway may allow synthesis of ATP from G6P. However, some of the ATP needed may have to be imported from the cytosol via an ATP/ADP translocator (AATP) located in the inner envelope membrane (Fig. 2.1) (Neuhaus *et al.*, 1997; Neuhaus and Emes, 2000). In the latter case, the glycolytic or respiratory origin of ATP has not been determined.

2.5.2.3 HK contributes to the production of NDP-sugars for various pathways

HK may contribute to the supply of NDP-sugars for synthesis of non-cellulosic cell wall polysaccharides, and glycosylation of glycoproteins and small lipophilic molecules (Fig. 2.1). In support of that view, NDP-sugar levels were diminished in maize root tips following inhibition of a mitochondrial HK (Galina and da-Silva, 2000). HK-derived G6P may be isomerized by phosphoglucomutase (PGM) to G1P, which can in turn be transformed into UDP-Glc by UDP-D-glucose pyrophosphorylase (UGPase). F6P may also feed the UDP-Glc pool after isomerization to G6P by phosphoglucose isomerase (PGI). Similarly, M6P may be isomerized by phosphomannomutase (PMM) to D-mannose-1-phosphate (M1P). The latter is then transformed into GDP-D-mannose (GDP-Man) by GDP-Man pyrophosphorylase (GMPase). GDP-Man is the precursor of L-ascorbate, which has major implications in cell wall synthesis, reactive O_2 species detoxification and, possibly, cell division (Conklin, 2001). Furthermore, UDP-Glc and GDP-Man are the two initial substrates of specific NDP-sugar interconverting enzymes (NIEs) that produce various UDP- and GDP-sugars (Seifert, 2004). Those most likely enter the endomembrane system via specific NDP-sugar transporters (NDPTs) to be used by glycosyltransferases involved in synthesis of glycoproteins and cell wall polysaccharides, e.g. pectins and hemicelluloses (Keegstra and Raikhel, 2001). Alternatively, the NDP-sugars may serve as donor substrates for glycosyltransferases that catalyze glycosylation of all hormones but ethylene, and secondary metabolites like phenylpropanoids or flavonoids (Bowles *et al.*, 2006). These glycosyltransferases may be cytosolic or associated with the cytosolic face of compartment membranes (Fig. 2.1) (Bowles *et al.*, 2006). Another class of

glycosyltransferases, localized in the chloroplast envelope membranes, is implicated in synthesis of glycolipids that compose chloroplastic and extraplastidic membranes (Benning and Ohta, 2005). The data presented above suggest that the HK reaction is involved in numerous major aspects of plant physiology by feeding G6P to a variety of metabolic pathways.

2.5.3 HK isoforms have different biochemical properties that suggest specific roles

2.5.3.1 Physico-chemical properties

Studies of HK at the protein level have unraveled the complexity of multiple HK isoforms in various plant organs and tissues. Although few of those have been assigned a coding gene, they could be distinguished on the basis of their subcellular localizations, and chromatographic and kinetic properties (Miernyk and Dennis, 1983; Doehlert, 1989; Schnarrenberger, 1990; Renz *et al.*, 1993; Renz and Stitt, 1993; da-Silva *et al.*, 2001). The subcellular localizations of HK isoforms will be discussed in a later section. As for their chromatographic properties, native plant HKs have been assigned molecular masses ranging from 38 to 68 kDa (Higgins and Easterby, 1974; Miernyk and Dennis, 1983; Doehlert, 1989; Renz *et al.*, 1993; Yamamoto *et al.*, 2000). This would correspond to monomers since HK cDNAs encode peptides with molecular masses of 54 kDa (Veramendi *et al.*, 2002; Giese *et al.*, 2005; Claeyssen *et al.*, 2006; Kandel-Kfir *et al.*, 2006). The deviation of M_r data from the value of 54 kDa may then suggest proteolytic degradation or behavior different from that of globular proteins in size exclusion chromatography.

Regarding their kinetic behavior, HKs from a given plant tissue may differ in their affinities for their preferential substrate, Glc (Doehlert, 1989; Renz and Stitt, 1993; da-Silva *et al.*, 2001). The K_m for Glc is generally low and may vary between 20 and 130 μM (Table 2.1). Affinity for Man is comparable, with a K_m ranging from 20 to 500 μM. By contrast, the K_m for Fru is always in the millimolar range (Table 2.1). HKs are known to display Michaelis–Menten kinetics with these sugar substrates (Higgins and Easterby, 1974; Doehlert, 1989; Schnarrenberger, 1990; Renz and Stitt, 1993; da-Silva *et al.*, 2001; Menu *et*

al., 2001; Claeyssen *et al.*, 2006). Glc is probably not limiting in leaves, where its cytosolic levels have been estimated at 100–400 µM, in tobacco and other dicotyledons (Leidreiter *et al.*, 1995; Moore *et al.*, 1997). In potato tuber, cytosolic Glc concentrations can reach 30 mM while those of Man and Fru are respectively more than three orders of magnitude lower and not detected (Farré *et al.*, 2001). Consequently, HK is likely to phosphorylate Glc almost exclusively in this heterotrophic organ. As for nucleoside triphosphates, in most cases HK has highest affinity for ATP, with a K_m between 50 and 590 µM (Table 2.1). Cytosolic concentrations above 200 µM have been measured for ATP in potato tuber (Farré *et al.*, 2001), suggesting that ATP is not limiting in heterotrophic cells. By contrast, the K_m (UTP) of HK (Table 2.1) was much higher than the estimate of UTP levels in the cytosol of potato tuber cells (Farré *et al.*, 2001). Therefore, it seems doubtful that UTP plays a significant role in hexose phosphorylation by HK in heterotrophic cells. Besides substrate affinity, HK sensitivity to pH changes may vary with the plant species or among isoforms of a given tissue. HKs may display broad or narrow response curves to pH, the latter with a pH optimum between 8 and 8.7 (Doehlert, 1989; Renz and Stitt, 1993; Veramendi *et al.*, 1999; Dai *et al.*, 2002; Claeyssen *et al.*, 2006). Selective expression of HK isoforms with contrasting kinetic properties may be a means for the cell to finely tune hexose catabolism depending on substrate and energy levels, or following pH changes. In support of that view, a rise in HK activity was correlated to the requirement for carbon flow through the Glc pool at specific stages of potato tuber development (Renz *et al.*, 1993). In particular, the kinetic properties of the different HK isoforms appeared of prime importance for their regulation *in vivo*, in relation to the physiological status of the tuber (Renz and Stitt, 1993). Additional regulatory properties, such as sensitivity to inhibition by ADP and G6P, are considered below as they may confer unique features to plant HKs.

Table 2.1: Kinetic constants of plant hexokinases for their substrates.

Substrate	K_m (mM)	References
D-glucose	0.02–0.13	[a, b, c, d, e, f, g, h, i, j, k, l, m]
D-fructose	1.5–30	[a, b, c, d, f, g, h, i, j, k, l, m]
D-mannose	0.02–0.5	[a, b, c, f, g, i, j, k, l]
ATP	0.05–0.59	[b, d, e, h, j, k, l]
UTP	1.3–4.5	[d, h, l]
GTP	0.75–2.2	[d, h, l]
CTP	1–3.2	[d, h, l]
TTP	0.23	[l]

References used are as follows: [a] Turner *et al.*, 1977; [b] Turner and Copeland, 1981; [c] Tanner *et al.*, 1983; [d] Renz and Stitt, 1993; [e] Galina *et al.*, 1995; [f] Veramendi *et al.*, 1999; [g] Wiese *et al.*, 1999; [h] da-Silva *et al.*, 2001; [i] Menu *et al.*, 2001; [j] Dai *et al.*, 2002; [k] Giese *et al.*, 2005; [l] Claeyssen *et al.*, 2006; [m] Kandel-Kfir *et al.*, 2006.

2.5.3.2 HK sensitivity to ADP inhibition

HK isoforms from different plant species and tissues (Table 2.2) are sensitive, to varying degrees, to inhibition by ADP (K_i of 0.03–1 mM). Furthermore, in maize roots, an HK bound to mitochondria or microsomal membranes was highly sensitive to inhibition by ADP whereas cytosolic HK was not (Table 2.2). ADP competed with neither hexose nor ATP, suggesting a distinct binding site for ADP (Galina *et al.*, 1995). As for soluble HKs partially purified from wheat germ and potato tuber, ADP was non-competitive inhibitor to Glc but acted competitively to ATP with a small non-competitive component (Higgins and Easterby, 1974; Renz and Stitt, 1993). These data may reflect differences between soluble and non-cytosolic HKs regarding the regulatory mechanisms underlying their inhibition by ADP (Galina *et al.*, 1995). Their contrasting sensitivities may have important repercussions on carbohydrate metabolism under energy-limited conditions such as hypoxia or anoxia.

During a switch from normoxia to anoxia, there is a rise in ADP levels, e.g. from 30 to 170 μM in maize roots (Hooks *et al.*, 1994), which may inhibit non-cytosolic HK significantly (Galina *et al.*, 1995). Nevertheless, the physiological significance of such preferential inhibition under anoxia remains to be clarified. In addition to ADP, mannoheptulose and glucosamine inhibited non-cytosolic HK activity to a far greater extent than cytosolic HK activity (Table 2.2). These effects were interpreted as evidence for a role of non-cytosolic HK in hexose and energy charge sensing, whereas cytosolic HK would carry out a catalytic function in glycolysis (Galina and da-Silva, 2000; da-Silva *et al.*, 2001). Although direct evidence is needed, these findings raise the interesting possibility that the regulatory properties and subcellular localizations of HK isoforms may be integrated in their individual roles.

2.5.3.3 Disparate sensitivities of HK isoforms to G6P inhibition

Plant HKs differ from mammalian HKs with respect to their sensitivities to G6P. Mammalian HKs I, II, and III are highly sensitive to inhibition by G6P (Wilson, 2003). This contrasts with the low sensitivity (K_i = 4 mM) or absence thereof, to near-physiological G6P levels for plant HKs (Table 2.2). Cytosolic concentrations of G6P have indeed been estimated at 6 mM in spinach leaves and 0.3 mM in potato tuber (Winter *et al.*, 1994; Farré *et al.*, 2001). Furthermore, sensitivity to G6P seems to vary between HK isoforms (Table 2.2). In potato tuber, activity of HK1 was inhibited by G6P whereas HK2 was insensitive (Renz and Stitt, 1993). When inhibition by G6P was observed in tomato and potato tuber, it was pH-sensitive (Table 2.2). The physiological significance of *in vivo* regulation of certain HK isoforms by G6P remains elusive (Renz and Stitt, 1993). In human (*Homo sapiens*) brain cells, G6P controls the binding of HK I to mitochondria, thereby regulating the relative levels of soluble and membrane-bound forms (Cárdenas *et al.*, 1998). In plants, G6P could solubilize mitochondrion-associated HK from castor bean endosperm, but not those from pea (*Pisum sativum*) stems and leaves (Miernyk and Dennis, 1983; Dry *et al.*, 1983; Tanner *et al.*, 1983). Altogether, these results suggest major differences between plant and mammalian HKs, and among plant HKs, with respect to G6P-mediated regulation.

2.5.3.4 Plant HKs are insensitive to D-trehalose-6-phosphate

Regulation of plant HKs may also differ substantially from that of yeast HKs, especially with respect to sensitivity to D-trehalose-6-phosphate (T6P), an intermediate of D-trehalose biosynthesis. In the yeast *S. cerevisiae*, mutant studies have implicated T6P in the control of glycolytic flux via inhibition of the most active HK isoform, HK PII (Thevelein and Hohmann, 1995). In plants, T6P has been implicated in regulation of major processes such as photosynthesis, carbohydrate utilization for growth, starch synthesis and seed maturation (Paul *et al.*, 2001; Schluepmann *et al.*, 2003; Kolbe *et al.*, 2005; Gomez *et al.*, 2006). By analogy to the yeast model, T6P has been proposed to control plant glycolysis through inhibition of HK (Paul *et al.*, 2001; Schluepmann *et al.*, 2003). However, to date, experimental evidence for inhibition of plant HK activity by T6P is still lacking (Table 2.2). Nonetheless, one cannot rule out the possibility that plants contain T6P-sensitive HKs until all members of their gene family have been characterized. The findings presented above suggest that kinetic and regulatory properties and subcellular localizations of HK isoforms may be of prime importance in their individual roles in plants. These observations also apply to mammalian HKs (Wilson, 2003). However, the peculiarity of plant HKs with respect to inhibition by G6P and T6P suggests that their modes of action may be unique among Eukaryotes.

Table 2.2: Effectors of hexokinase activity.

Effector	Species, Tissue, HK isoform	K_i or % inhibition with D-glucose as substrate	References
ADP [a]			
	T. aestivum, germ HK	K_i = 1 mM	[a]
	P. sativum, seed, HK I and II	50% at ~1 mM	[b, c]
	Z. mays, kernel, HK1 and HK2	45% at 1 mM	[d]
	Z. mays, root, non-cytosolic HK	K_i = 0.03 mM	[f, g, i]
	cytosolic HK	no effect	[f, g, i]
	S. lycopersicum, recombinant LeHxk1 to LeHxk4	~50% at 5 mM	[j, k]
	S. tuberosum, tuber, HK1	K_i = 0.04 mM	[e]
	HK2	K_i = 0.11 mM	[e]
	S. chacoense, recombinant ScHK2	60% at 0.1 mM	[l]
D-glucose-6-phosphate			
	P. sativum, seed, HK I	no effect	[b]
	Z. mays, kernel, HK1 and HK2	weak inhibition	[d]
	Z. mays, root, non-cytosolic HK	no effect	[f]
	cytosolic HK	no effect	[f]
	S. lycopersicum, recombinant LeHxk2	20% at 5 mM (pH 7)	[j]
	S. tuberosum, tuber, HK1	K_i = 4 mM at pH 7	[e]
	HK2	no effect	[e]
	S. chacoense, recombinant ScHK2	no effect (pH 7, pH 8)	[l]
glucosamine or *N*-acetylglucosamine [b]			
	Z. mays, root, non-cytosolic HK	K_i = 0.8 mM	[i]
	cytosolic HK	K_i = 80 mM	[i]
	S. lycopersicum, recombinant LeHxk2	20% at 50 mM	[j, k]
	recombinant LeHxk3 and LeHxk4	20% at 15 mM	[j, k]

S. chacoense, recombinant ScHK2	50% at 5 mM	[l]
S. oleracea, leaf, SoHxK1	K_i = 50 mM	[m]
mannoheptulose [b]		
Z. mays, root, non-cytosolic HK	K_i = 0.5 mM	[h, i]
cytosolic HK	K_i = 20 mM	[h, i]
S. lycopersicum, recombinant LeHxk2	20% at 50 mM	[j]
D-trehalose-6-phosphate		
S. lycopersicum, recombinant LeHxk1 to LeHxk4	no effect	[k]
S. chacoense, recombinant ScHK2	no effect	[l]
S. oleracea, leaf, SoHxK1	no effect	[m]
A. thaliana, leaf, AtHXK1 and AtHXK2	no effect	[n]

[a] Possible chelation effects of ADP were taken in account in [a], [c], [d], [e], [f], [g], [i] and [l] to ensure that HK activity inhibition was due to ADP and not to Mg^{2+} chelation.

[b] Glucosamine, *N*-acetylglucosamine and mannoheptulose have been used to demonstrate the implication of *Arabidopsis* HK in sugar sensing (Jang and Sheen, 1994; Jang *et al.*, 1997).

HK isoforms were named as in the articles cited. References are as follows: [a] Higgins and Easterby, 1974; [b] Turner *et al.*, 1977; [c] Turner and Copeland, 1981; [d] Doehlert, 1989; [e] Renz and Stitt, 1993; [f] Galina *et al.*, 1995; [g] Galina *et al.*, 1999; [h] Galina and da-Silva, 2000; [i] da-Silva *et al.*, 2001. Note: non-cytosolic HK is associated with mitochondrial, Golgi or microsomal fractions; [j] Menu *et al.*, 2001; [k] Kandel-Kfir *et al.*, 2006; [l] Claeyssen *et al.*, 2006; [m] Wiese *et al.*, 1999; [n] Eastmond *et al.*, 2002.

2.6 On-line molecular data raise intriguing questions about the roles and modes of action of HK isoforms

2.6.1 HK distribution at the organismic level demonstrates a complex regulation and suggests that HK isoforms have tissue- and stress-specific roles

2.6.1.1 The Arabidopsis *genome expresses six HK genes*

The existence of multiple HK isoforms in plants raises questions about their specificities to certain tissues, and their individual roles in cells where they occur. These questions led us to examine expression profiles of HK genes from *Arabidopsis*, for which DNA microarray data are publicly accessible. BLAST searches of the *Arabidopsis* genome (The Arabidopsis Initiative, 2000) were carried out, using *At4g29130* and *At2g19860* nucleotide sequences, and amino acid sequences of their respective protein products AtHXK1 and AtHXK2 (The Institute for Genomic Research website, http://www.tigr.org/tdb/e2k1/ath1/). Four uncharacterized HK-like expressed sequence tags (ESTs) were identified: *At1g47840*, *At1g50460*, *At3g20040* and *At4g37840*. Multiple alignment of the six HK sequences illustrates that they have 66 to 96% sequence homology (Supplementary Fig. 2.1). These data support the view that the various HK isoforms of one organism have significant sequence homology. A UniProt entry, named T2E6.5 (Q9FZG4), is also available at the Expert Protein Analysis System (ExPASy) website (http://ca.expasy.org/) (Gasteiger *et al.*, 2003). However, this genomic sequence has no corresponding EST. It encompasses *At1g47840* and contains additional regions homologous to HK sequences. These regions are apparently not expressed, based on EST search in the *Arabidopsis* Tiling Array Transcriptome (http://signal.salk.edu/) (Yamada *et al.*, 2003). It is possible that these regions of T2E6.5 have arisen from a partial duplication of an HK sequence.

2.6.1.2 The expression patterns of the six HK genes of Arabidopsis *are not correlated at the entire plant level*

The expression profiles of *AtHXK1*, *AtHXK2*, *At1g47840*, *At1g50460*, *At3g20040* and *At4g37840* (Fig. 2.2) were obtained from two data sets available on-line: the AtGenExpress Consortium Tissue and Stress series (Botany Array Resource, http://bbc.botany.utoronto.ca/) (Toufighi *et al.*, 2005). The six HK genes did not seem to be subject to co-regulation at the whole plant level or in response to abiotic stresses (Fig. 2.2A and B). At best, *At4g37840* and *AtHXK1* exhibited a moderate correlation in expression patterns in the entire plant, with a Pearson's correlation coefficient of 0.67 (Expression Angler tool, http://bbc.botany.utoronto.ca/). A perfect correlation between two genes would lead to a coefficient of 1. Correlation coefficients were lower for all other HK sequences in the Tissue and Stress data sets (data not shown).

2.6.1.3 Some HK isoforms may have tissue-specific roles in plants

At1g47840 was highly expressed in root and seed (Fig. 2.2A), which may reflect a specific role of its protein product in these systems. In the seed, *At1g47840* expression peaked during heart to late torpedo stages (Fig. 2.2A, seed stages 4–7 described by Schmid *et al.*, 2005). This corresponded to the end of embryo morphogenesis, the first phase of *Arabidopsis* seed development, characterized by cell divisions. The second phase, embryo maturation, consists of cell expansion and accumulation of storage proteins and lipids in the embryo. Transient starch synthesis and degradation takes place at the transition between these two phases (Baud and Graham, 2006). Cell division during heart to torpedo stages in the *Arabidopsis* seed has been correlated to a high hexose/Suc ratio, which decreased during subsequent cell elongation and lipid storage (Baud *et al.*, 2002). Consequently, the aforementioned statement that a high hexose/Suc ratio favors cell division during sink initiation (Weber *et al.*, 1997), may apply to the *Arabidopsis* seed (Baud *et al.*, 2002). Furthermore, expression of *At1g47840* peaked at these stages (Fig. 2.2A), suggesting that the At1g47840 protein product may support cell division in some way. However, total HK activity has been shown to rise three to four days later, during the maturation phase (Focks

and Benning, 1998; Baud and Graham, 2006). Uncorrelated levels of *At1g47840* transcript and HK activity suggest that some regulation may take place at the posttranscriptional and/or posttranslational levels, and that the catalytic activity of At1g47840 may not be involved in its function. It may then be proposed that At1g47840 supports cell division as a hexose sensor, a function discussed later.

It was observed elsewhere that a peak of HK activity coincided with starch degradation and storage lipid deposition during seed development in *Arabidopsis* (Baud and Graham, 2006). This may suggest an additional role for HK in directing carbon flow from Suc or transitory starch-derived Glc to lipid synthesis. In support of that view, incorporation of Glc into seed lipids was compromised in the *Arabidopsis* mutant *wrinkled1* that lacked HK activity (Focks and Benning, 1998). Furthermore, the onset of the SuSy path of Suc cleavage during the maturation phase coincided with a shift from ATP-dependent activities to pyrophosphate (PP_i)-consuming activities (Baud and Graham, 2006). This switch to more energy-efficient reactions is viewed as an adaptive response to the low O_2 tensions that limit ATP production in developing oilseeds (Rolletschek *et al.*, 2005; Baud and Graham, 2006). Nonetheless, HK activity displayed the same temporal pattern as SuSy activity rather than decreasing like the other ATP-dependent enzyme activities measured (Baud and Graham, 2006). Thus, the data may indicate an absolute requirement for HK in storage lipid deposition. Only the *AtHXK2* gene was slightly induced during the rise in total HK activity, the other HK genes were unaffected or even repressed, like *AtHXK1* (Fig. 2.2A; Baud and Graham, 2006). Again, the results strongly suggest posttranscriptional and/or posttranslational regulation of HK activity during seed development. Moreover, the question as to which HK isoform(s) may contribute G6P for seed lipid synthesis remains unresolved. The *Wrinkled1* gene has been found to code for a transcription factor, WRI1, which regulates expression of some glycolytic genes (Cernac and Benning, 2004). WRI1 is likely to impact on HK activity since the latter was abolished in the *wrinkled1* mutant (Focks and Benning, 1998). However, mRNA levels of the six HK genes were similar in wild-type and *wrinkled1* seeds, suggesting that WRI1 does not modulate HK gene transcription (Ruuska *et al.*, 2002). Collectively, these findings suggest

that HK is subject to posttranscriptional and/or posttranslational regulation in the seed, but the regulatory mechanisms involved remain unknown.

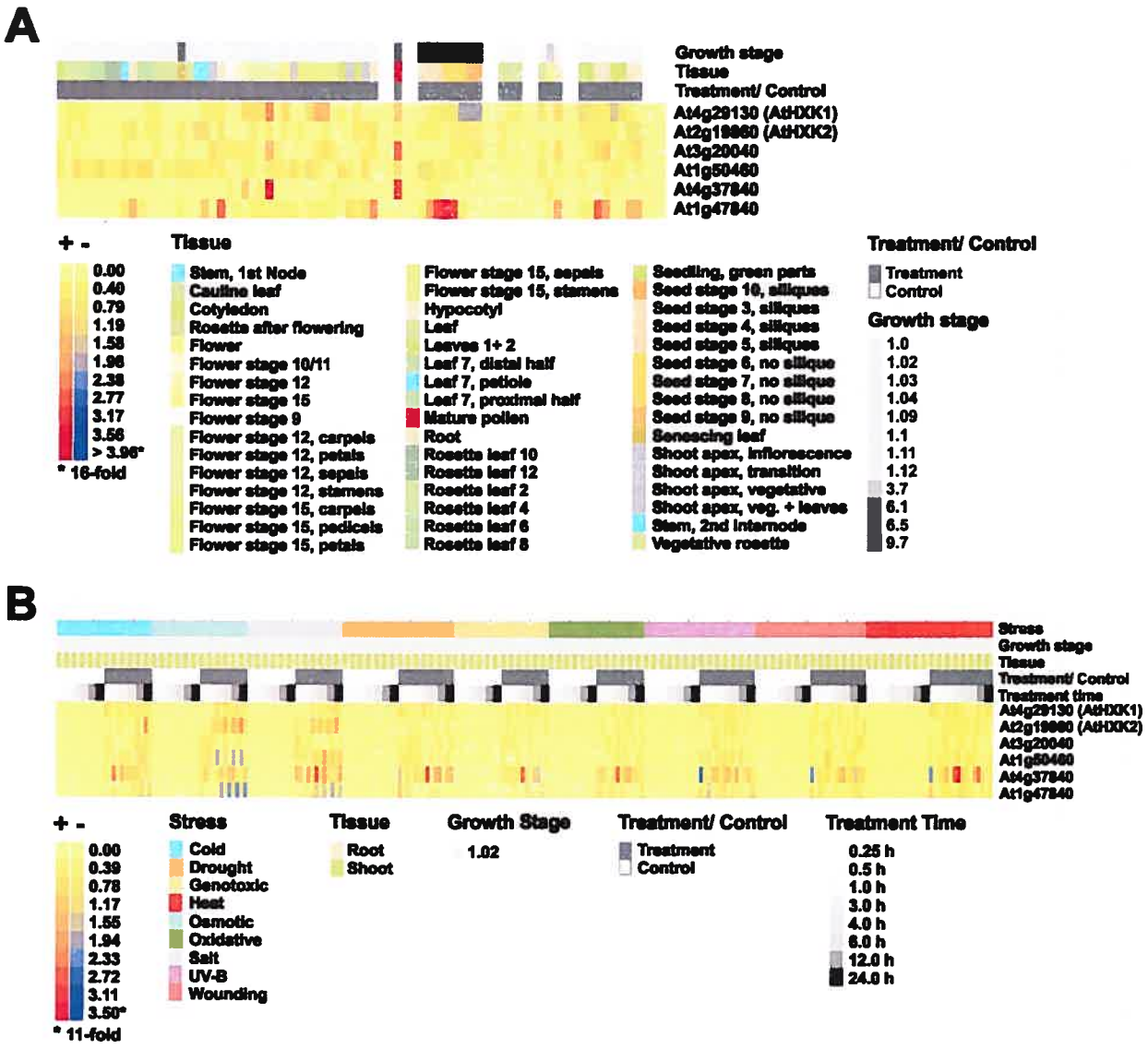


Figure 2.2: Expression profiles of the six hexokinase (HK) genes of *Arabidopsis*, in the different plant parts (A) and in response to abiotic stresses (B), using the Expression Browser on Affymetrix ATH1 array data from the AtGenExpress Consortium Tissue and Stress series, respectively (http://bbc.botany.utoronto.ca/).

The HK sequences were named after their AGI numbers: *At4g29130* (*AtHXK1*), *At2g19860* (*AtHXK2*), *At3g20040*, *At1g50460*, *At4g37840* and *At1g47840*. Each gene of the AtGenExpress Tissue series was considered as a 'treatment' and compared to a median value ('control') calculated across all wild-type samples in the data set used by Schmid *et al.* (2005). As for the AtGenExpress Stress series, stress treatments were stored as separate projects, each with a corresponding wild-type for control. The color scale indicates $\log_2$-transformed levels of expression above (red) or below (blue), the control (yellow) (Toufighi *et al.*, 2005). Values are means of three independent expression estimates from triplicate Affymetrix ATH1 arrays (Schmid *et al.*, 2005). Growth stages are depicted in Supplementary Table 2.2 according to Boyes *et al.* (2001). Seed stages are described further by Schmid *et al.* (2005). The reader is referred to the above-cited website for details on procedures for each microarray experiment.

In Fig. 2.2A, *At3g20040*, *AtHXK1* and *At4g37840* were expressed at highest levels in stamen and pollen grain whereas *At1g50460* was strongly repressed. This suggests specific roles for At3g20040, AtHXK1 and At4g37840 protein products in male reproductive tissues. In particular, pollen development relies on Suc import from the apoplast and its hydrolysis by cwINV (Goetz *et al.*, 2001). It may, therefore, be proposed that mitosis, or starch or lipid reserve synthesis during subsequent pollen maturation (Clement *et al.*, 1994), relies on utilization by HK of hexoses arising from Suc hydrolysis. By contrast, all six ESTs were expressed at rather basal levels in other plant parts (Fig. 2.2A). Similar observations have been made recently with rice (*Oryza sativa*) plants, which expressed one pollen-specific and eight ubiquitous HK genes (Cho *et al.*, 2006). In tomato, also, *LeHXK2* and *LeHXK3* were found at low levels in flower and in root and apex, respectively, whereas *LeHXK1* and *LeHXK4* were ubiquitously and highly expressed (Kandel-Kfir *et al.*, 2006). However, gene expression constitutes only one of several levels at which regulation can occur, as exemplified by HK gene and activity data from the developing seed. Unfortunately, HK regulation at the protein and activity levels and the mechanisms involved, have been largely ignored in plants. Above all, the link has often been missing between HK genes and their protein products and corresponding activities for the vast majority of plant organs investigated. It is thus conceivable that HK activity in some plant organs may result from a single or limited number of isoforms. In support of that view, a stromal HK (GenBank AY260967) was shown to be the main Glc-phosphorylating enzyme in the moss *Physcomitrella patens*, accounting for 80% of total activity in protonemal tissue (Olsson *et al.*, 2003). A similar organ/tissue preponderance has been documented in other eukaryotic organisms. In rat (*Rattus norvegicus*), HK I is expressed at high levels in brain, whereas HK II predominates in muscle, and HK IV in hepatocytes and pancreatic β-islets. It was noticed that their kinetic properties made these HK isoforms particularly suited to their presumed roles in their respective locations (Cárdenas *et al.*, 1998). In plant HK research, the transcriptomic data need to be complemented with activity profiles in order to determine which HK isoforms contribute to total HK activity. Examination of their kinetic properties may then help to elucidate their individual roles.

2.6.1.4 Some HK isoforms may have stress-specific roles in plants

As for responses to abiotic stresses, ESTs *At1g47840*, *At1g50460*, *AtHXK2* and *At4g37840* clearly exhibited responsive expression patterns (Fig. 2.2B). The *At4g37840* gene was the most responsive in all stress treatments: genotoxic stress by bleomycin and mytomycin, cold, drought, heat, salt, UV-B, wounding, osmotic and oxidative stress. Its expression peaked within the first 3 h of treatment, sometimes followed by repression compared to the control. Most pronounced effects on expression of *At4g37840* were observed under heat, drought, salt and UV-B stress, with generally greater amplitude in the root than in the shoot (Fig. 2.2B). In response to cold, osmotic and salt stress, *At1g47840* and *At1g50460* were consistently repressed in the root and in the shoot, respectively, and after as little as 1 h of treatment. On the contrary, *AtHXK2* was clearly induced in shoot and root within 3 h of osmotic and salt stress (Fig. 2.2B). Accordingly, *AtHXK2* was found to be induced 2–3-fold under cold, osmotic and salt stress in published microarray experiments (Kreps *et al.*, 2002). In addition, significant induction of *AtHXK2* by chilling was observed in wild-types but not in chilling-lethal mutants, suggesting a role for AtHXK2 in acclimation to suboptimal temperatures (Provart *et al.*, 2003). Hypoxia is another stress that significantly induced *AtHXK2*, together with *At1g47840* (Liu *et al.*, 2005; Loreti *et al.*, 2005). On the contrary, *At4g37840* was dramatically repressed (Loreti *et al.*, 2005). It is worth noting that HK helped sustain high glycolytic activity in maize root tips during hypoxic acclimation, thereby improving their survival under low O_2 tensions (Bouny and Saglio, 1996). Taken together, these data suggest that the HK isoforms encoded by the *At1g47840*, *At1g50460*, *AtHXK2* and *At4g37840* genes may be implicated in specific stress responses. Unfortunately, HK is not often mentioned in proteome profiling studies. This may not contradict the data cited above, but rather point to HK as a low abundance protein not easily detectable in complex protein mixtures. It should then be made clear that variations in activity of a protein that escapes detection in large profiling studies may still impact dramatically on metabolism.

2.6.2 HK distribution at the subcellular level

2.6.2.1 HKs can be soluble in the cytosol or associated with organelles

It has already been mentioned that HK isoforms could be distinguished on the basis of their subcellular localizations. Several studies have shown that a given tissue could contain HK isoforms localized to different cell compartments. In maize roots, active HK isoforms have been found in cytosolic, Golgi and mitochondrial fractions (da-Silva *et al.*, 2001). Cytosolic, mitochondrial and plastidic HK isoforms have been characterized in developing castor oil seeds (Miernyk and Dennis, 1983). Soluble and mitochondrial HKs have also been partially purified from pea and spinach leaves (Baldus *et al.*, 1981; Schnarrenberger, 1990). In pea roots, plastid-associated HK accounted for 16% of total HK activity (Borchert *et al.*, 1993), suggesting the presence of other HK isoforms in other cell compartments. The possibility of cross-contaminations between purified fractions cannot be dismissed for these studies. Nevertheless, several HKs identified by their coding gene, have been localized *in situ* and could be distinguished from HK activities in other cell compartments. Two HK isoforms, At1g50460 and AtHXK2 (At2g19860), have been identified on the outer mitochondrial membrane of *Arabidopsis* suspension cells (Giegé *et al.*, 2003). AtHXK1 (At4g29130) transfected in maize protoplasts has been found in nuclear fractions (Yanagisawa *et al.*, 2003), thus suggesting different subcellular locations for the different HK isoforms of *Arabidopsis*. In spinach leaves, the SoHxK1 isoform anchored in the chloroplastic outer envelope membrane was distinct from soluble HK activity, based on immunodetection evidence (Wiese *et al.*, 1999). An HK has been localized in the plastid stroma in various tissues of the moss *Physcomitrella patens*, tobacco, tomato and rice. Other HK isoforms were identified, or their activity measured, in other cell compartments in these cases (Olsson *et al.*, 2003; Giese *et al.*, 2005; Kandel-Kfir *et al.*, 2006; Damari-Weissler *et al.*, 2006; Cho *et al.*, 2006).

2.6.3 Importance of the N-terminus in HK targeting

The N-terminus of several plant HKs has been implicated in their targeting to specific cell compartments (Wiese *et al.*, 1999; Olsson *et al.*, 2003; Giese *et al.*, 2005; Claeyssen *et al.*, 2006). This has led us to examine the N-terminal sequences of HKs from *Arabidopsis*, several *Solanaceae*, spinach and the moss *Physcomitrella* (Fig. 2.3). In each species, one HK shared sequence homology with the stromal HK from *Physcomitrella*, PpHxk1, thus constituting the type A group according to Olsson *et al.* (2003). These HKs were predicted to contain a transit peptide and to be targeted to the chloroplast, using the ChloroP 1.1 and TargetP 1.1 programs available at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/) (Emanuelsson *et al.*, 2000). The stromal localization of PpHxk1, NtHxk2 and LeHxk4 has been confirmed experimentally (Olsson *et al.*, 2003; Giegé *et al.*, 2003; Kandel-Kfir *et al.*, 2006; Damari-Weissler *et al.*, 2006). Using the SignalP 1.1 program from the same website, the N-termini of type B were predicted to contain a signal peptide absent from animal and yeast HKs (Fig. 2.3) (Olsson *et al.*, 2003). Most were predicted to be targeted to the secretory pathway except PpHxk2, which differed significantly in sequence and was predicted to be targeted to the chloroplast (Fig. 2.3). Some type B HKs have been shown to be anchored in, or associated with, membranes (Wiese *et al.*, 1999; Giegé *et al.*, 2003; Kandel-Kfir *et al.*, 2006, Damari-Weissler *et al.*, 2006). Some contain a prokaryotic attachment site in their N-terminus that may play a role in interactions with membranes (Giegé *et al.*, 2003). Also, type B HKs typically display electropositive surfaces near their membrane anchor domain (Fig. 2.3) that may enhance their association with negatively charged membrane components (Kandel-Kfir *et al.*, 2006). Lastly, SoHxK1 was inserted into the outer chloroplast membrane via its N-terminal membrane anchor, without need for proteinaceous receptors or ATP (Wiese *et al.*, 1999). Despite these clues, however, the mechanisms of insertion/binding to membranes are still unclear.

Interestingly, different subcellular localizations have been reported for type B HKs, sometimes for a same HK isoform. We recently characterized a putative plasma membrane-anchored HK (Fig. 2.1) from *Solanum chacoense*, ScHK2 (Claeyssen *et al.*, 2006). In tomato, LeHxk1, LeHxk2 and LeHxk3 have been associated with mitochondrial

membranes (Kandel-Kfir *et al.*, 2006; Damari-Weissler *et al.*, 2006). In *Arabidopsis*, At1g50460, AtHXK1 (At4g29130) and AtHXK2 (At2g19860) have been associated with the mitochondrion (Giegé *et al.*, 2003; Rolland *et al.*, 2006). However, AtHXK1 has also been found in the nucleus (Yanagisawa *et al.*, 2003). As for spinach, SoHxK1 was localized not only in the outer chloroplast membrane but also in mitochondrial fractions, as shown by HK activity assay and immunodetection (Wiese *et al.*, 1999). It is not clear whether or not SoHxK1 was present in a peak of HK activity that co-fractionated with that of a Golgi marker after isopycnic sucrose gradient centrifugation (Wiese *et al.*, 1999). If so, this would suggest a localization of SoHxK1 in endomembranes. It is thus possible that plant HKs are subject to dual targeting and/or translocation events. Dual targeting of proteins to mitochondria and chloroplasts has been documented in several instances in plants (Peeters and Small, 2001; Cleary *et al.*, 2002; Duchêne *et al.*, 2005). Also, the protein disulfide isomerase RB60 was targeted to the chloroplast and the endoplasmic reticulum (ER) in the green alga *Chlamydomonas reinhardtii* (Levitan *et al.*, 2005). Several dual-targeted proteins have been found to contain two targeting signals in their N-terminus, either in tandem or overlapping (Chabregas *et al.*, 2001; Chew *et al.*, 2003; Levitan *et al.*, 2005). In a similar manner, the N-terminus of type B HKs could have dual-targeting properties not recognized by prediction programs like TargetP 1.1. However, this hypothesis needs to be tested experimentally.

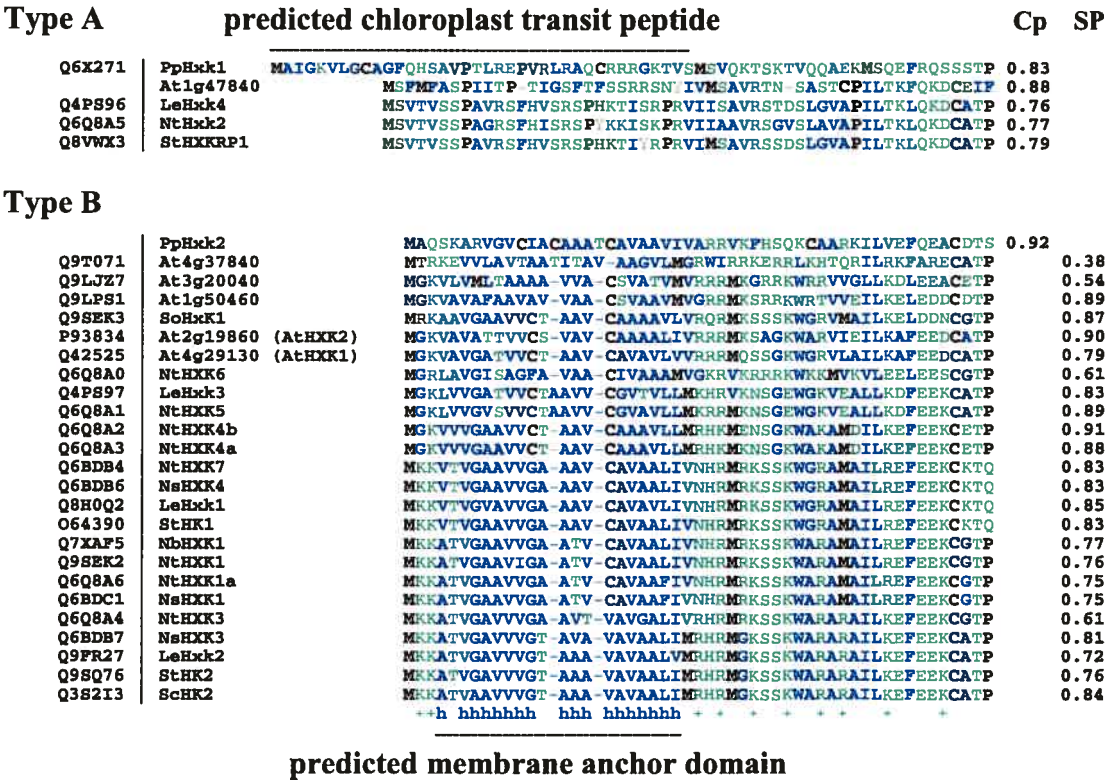


Figure 2.3: Multiple alignment of N-terminal sequences of hexokinases from five plant species, and their predicted targeting to the chloroplast (Cp) or to the secretory pathway (SP), using the TargetP 1.1 program.

Type A is hexokinases (HKs) with predicted chloroplast transit peptides, using the ChloroP 1.1 program. Type B is HKs with N-terminal membrane anchors, using the SignalP 1.1 program. All programs are available at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/) (Emanuelsson *et al.*, 2000). Hydrophobic (h) amino acids are represented in blue, polar, positive or basic (+) amino acids are in green, others are in black. UniProt entries of HKs are indicated on the left, followed by their AGI numbers or trivial names, some of which were taken from the following references: AtHXK1 and AtHXK2 (Jang *et al.*, 1997), LeHxk1 to LeHxk4 (Kandel-Kfir *et al.*, 2006), NtHxk2 (Giese *et al.*, 2005), PpHxk1 and PpHxk2 (Olsson *et al.*, 2003), ScHK2 (Claeyssen *et al.*, 2006), SoHxK1 (Wiese *et al.*, 1999), StHK1 and StHK2 (Veramendi *et al.*, 1999; Veramendi *et al.*, 2002). TargetP scores and predictions are shown on the right. Abbreviations indicate the species: At, *Arabidopsis thaliana*; Le, *Lycopersicon esculentum* (*Solanum lycopersicum*); Nb, *Nicotiana benthamiana*; Ns, *Nicotiana sylvestris*; Nt, *Nicotiana tabacum*; Pp, *Physcomitrella patens*; Sc, *Solanum chacoense*; So, *Spinacia oleracea*; St, *Solanum tuberosum*. The N-terminal sequence of PpHxk2 was taken from Olsson *et al.* (2003), that of StHK1 from Menu *et al.* (2001).

2.6.3.1 Are some HKs translocated between cell compartments?

Another possible cause for such diverse locations of type B HKs may be their translocation between different membrane systems. In mammals, translocation of some HK isoforms may have functional significance, as well as their binding to actin microfilaments (Pedley *et al.*, 1993; Murata *et al.*, 1997). In *Arabidopsis*, one may ask whether AtHXK1 is translocated between the mitochondrion and the nucleus, and how this may relate to its function in Glc signaling (Rolland *et al.*, 2006). If AtHXK1 is translocated, its binding to unknown molecules may be required as no nuclear targeting motif has been found in its gene sequence (ScanProsite tool, http://ca.expasy.org/). A good candidate may then be the actin cytoskeleton, which has been involved in the function of AtHXK1 in Glc signaling (publication 501716579 by Balasubramanian *et al.*, 2005, at http://www.arabidopsis.org/). Accordingly, actin bundles have been proposed to function in protein import from the cytoplasm into the plant nucleus (Collings *et al.*, 2000). Furthermore, AtHXK1 is found in a high-molecular mass protein complex in the nucleus (Fig. 2.1) (Rolland *et al.*, 2006), which may also be involved in regulation and/or translocation of AtHXK1. Therefore, study of interactions of AtHXK1 with the actin cytoskeleton and the nuclear protein complex may help determine whether translocation is part of its mode of action.

On the question of translocation of type B HKs, their possible association with stromules (stroma-filled tubular extensions of the plastid envelope) also deserves some consideration. In roots, plastids are abundantly interconnected by stromules (Kwok and Hanson, 2004). They have also been found to connect plastids with the nucleus or the plasma membrane in non-green tissues of *Arabidopsis* and tobacco (Kwok and Hanson, 2004). Stromules have been proposed to facilitate coordination of plastid activities by allowing the transport of stromal molecules between plastids (Köhler *et al.*, 1997). Notably, the stromal HK LeHxk4 has been localized in stromules (Kandel-Kfir *et al.*, 2006). It would be interesting to examine whether this bridge between organelles may also allow for trafficking of their membrane components.

Lastly, the exchange of membrane material between plant organelles may be more widespread a phenomenon than usually thought. Some domains of the ER have been

observed in close contact with the mitochondrion, the plasma membrane and the plastid (Staehelin, 1997; Benning *et al.*, 2006). There has been evidence to show or strongly suggest an exchange of membrane lipids at such apposition sites between the ER and these organelles (Staehelin, 1997; Benning *et al.*, 2006). Furthermore, some unicellular algae contain complex plastids with four membranes, the outermost being contiguous with the ER (Nassoury and Morse, 2005). Some preproteins targeted to these plastids are inserted into the ER membrane before being translocated to the plastid via vesicles of the secretory pathway (Nassoury and Morse, 2005). Therefore, it is not inconceivable that membrane-associated HKs may be exchanged between organelles according to similar mechanisms, or between organelles that are connected via stromules. Much remains to be discovered about the mechanisms underlying HK targeting to specific cell compartments or their possible translocation in plant cells. Their elucidation, together with detailed localization of all HK isoforms of a cell, will certainly help unravel their individual roles and modes of action.

2.7 Functions of plant HKs

2.7.1 Catalytic function in hexose metabolism

2.7.1.1 Evidence for a crucial role in regulation of primary metabolism

HK is best known as a glycolytic enzyme, therefore its catalytic function will be discussed first. The central role of glycolysis in plant metabolism has been an incentive for elucidating its regulation. However, its compartmentalization and structure as a network of alternative reactions (Plaxton, 1996) make it difficult to assess the contribution of individual enzymes, such as HK, on overall glycolytic flux. In animals, HK has been shown to exert a high level of control over glycolytic flux. Thus, values of flux control coefficients between 0.7 and 1 have been measured in mammalian erythrocytes, liver, heart, insulinoma and muscle cells (Rapoport *et al.*, 1974; Torres *et al.*, 1988; Kashiwaya *et al.*, 1994; Wang and Iynedjian, 1997; Puigjaner *et al.*, 1997; Jannaschk *et al.*, 1999). In plants, however, the control coefficient of HK over glycolytic flux has not been assessed. Nonetheless, indirect evidence may suggest a crucial role for HK in regulation of primary metabolism. HK

overexpression in tomato plants impaired growth and photosynthesis, and induced rapid senescence in photosynthetic tissues (Dai *et al.*, 1999). It also resulted in reduced size of tomato fruit and seed, possibly due to an excessively low ATP/ADP ratio for starch synthesis (Menu *et al.*, 2004). One of HK's products, G6P, is a potent activator of PEP carboxylase (PEPC) (Plaxton, 1996). It may then be proposed that HK activity could modulate flux through PEPC, an enzyme involved in the replenishment of the tricarboxylic acid cycle intermediates. By the same token, PEPC activity raises the P_i/PEP ratio, which activates ATP-dependent phosphofructokinase (PFK) and raises overall glycolytic flux (Plaxton, 1996). Another possible regulatory mechanism is the implication of HK in the Suc and Glc/Glc-P cycles that may increase energy demand, thus contributing to elevated glycolytic rate (Fernie *et al.*, 2002a; Urbanczyk-Wochniak *et al.*, 2003; Alonso *et al.*, 2005). Interestingly, G6P and, to a lesser extent, M6P and F6P, inhibit the activity of a SNF1-related protein kinase 1 (SnRK1) in spinach (Toroser *et al.*, 2000). SnRK1s are thought to function broadly in carbon partitioning and hexose signaling through regulation of enzyme activity and gene expression (Rolland *et al.*, 2006). In particular, two spinach SnRK1s have been found to phosphorylate SPS and nitrate reductase (NR) (Sugden *et al.*, 1999). Since SPS participates in Suc cycling (Nguyen-Quoc and Foyer, 2001), inhibition of SnRK1 by G6P may result in enhanced Suc cycling activity. It follows that G6P accumulation may facilitate its own catabolism through the glycolytic pathway by acting indirectly on both PEPC and SPS activities. G6P may also alleviate SnRK1-mediated inhibition of NR activity, thereby promoting nitrogen assimilation. Taken together, these results suggest that HK-derived G6P may help sustain glycolytic rate and activate both NR and PEPC, thereby coordinating carbon and nitrogen supplies for amino acid synthesis.

2.7.1.2 Integration of location and function for HK isoforms

As suggested earlier, the subcellular localizations of HK isoforms may relate to their individual roles. Putatively plasma membrane-anchored ScHK2 from *S. chacoense* has been proposed to facilitate hexose import into sink cells by maintaining a gradient of concentration across the plasma membrane (Claeyssen *et al.*, 2006). ScHK2 activity was highly sensitive to inhibition by ADP (Table 2.2), suggesting that hexose import may rely

on adequate energy charge for further metabolism into glycolysis (Claeyssen *et al.*, 2006). In spinach leaves, outer chloroplast membrane-anchored SoHxK1 has been proposed to energize Glc that exits the chloroplast during transitory starch degradation. SoHxK1 would help maintain a gradient of Glc across the chloroplast envelope, thereby facilitating its export (Wiese *et al.*, 1999; Weber *et al.*, 2000). Plants may also be equipped with a stromal HK that generates G6P following degradation of transitory starch to Glc (Giese *et al.*, 2005). The resulting accumulation of G6P in the stroma of guard cells may contribute to increasing turgor for stomatal opening (Ritte and Raschke, 2003). In principle, adjusting the relative activities of cytoplasmic and stromal HKs may be a means for the cell to regulate Glc traffic across the plastid envelope (Olsson *et al.*, 2003).

The localization of HK isoforms may be key to their individual roles by allowing privileged access to ATP supply. Thus, the chloroplast envelope-anchored HK has been claimed to use preferentially chloroplastic ATP in spinach leaf cells (Stitt *et al.*, 1978; Wiese *et al.*, 1999). Similarly, plant HK bound to the mitochondrial membrane has been proposed to gain facilitated access to mitochondrial ATP, while efficiently recycling ADP to sustain oxidative phosphorylation (Dry *et al.*, 1983; Galina and da-Silva, 2000; Yamamoto *et al.*, 2000). This is well established for mammalian cells with high energy needs, e.g. brain or cancer cells, where HK I bound to actively-respiring mitochondria is known to use intramitochondrial rather than cytosolic ATP as substrate (Wilson, 2003). Such substrate specificity of HK I is thought to facilitate coordination between glycolytic and respiratory activities, thus adapting the overall rate of Glc metabolism to energy demand in mammalian cells (Wilson, 2003). HK I binds to the voltage-dependent anion channel (VDAC) and to an adenylate translocator at contact sites between outer and inner mitochondrial membranes, in a G6P-sensitive manner (Wilson, 2003). Consequently, the rate of G6P utilization in mammalian cells may impact on glycolytic and respiratory rates by governing, at least partly, the extent to which HK I is bound to mitochondria (Wilson, 2003). In plants, the presence of an intact glycolytic sequence on the cytosolic face of mitochondria may ensure a direct supply of pyruvate to support mitochondrial respiration (Giegé *et al.*, 2003). Therefore, there may be some coordination between cytoplasmic glycolysis and mitochondrial respiration in plant systems. However, we mentioned

previously that HK binding to the mitochondrion was not always sensitive to G6P in plants (Dry *et al.*, 1983; Tanner *et al.*, 1983). The mechanisms involved thus require further investigation as they may differ greatly from those in mammals. Notably, sensitivity of plant HK isoforms to ADP inhibition may be of prime importance for their individual roles. In contrast with the cytosolic isoform, mitochondrial membrane-bound HK has proven highly sensitive to ADP inhibition in maize roots (Galina *et al.*, 1995; Galina and da-Silva, 2000). Blockage of NDP-sugar formation by ADP has led to the proposal that this isoform feeds G6P to energy-demanding pathways only under an adequate ATP/ADP ratio, thus acting as an energy charge sensor in plant cells (Galina and da-Silva, 2000). Under a low ATP/ADP ratio, ATP use may be restricted to cytosolic HK for cell metabolic maintenance as this HK activity is less affected by ADP inhibition (Galina and da-Silva, 2000). Upon return to a sufficiently high ATP/ADP ratio, putative plasma membrane-anchored HK may resume hexose import and commitment to glycolysis (Claeyssen *et al.*, 2006). Mitochondrial HK may also be relieved from ADP inhibition, resuming production of G6P for energy-costly biosyntheses of NDP-sugars and cell wall polysaccharides (Galina and da-Silva, 2000). These considerations also raise intriguing questions about which HK isoforms may contribute to Suc and Glc/Glc-P cycles. The recruitment of ADP-sensitive HK isoforms may lead to self-regulatory substrate cycles as their impact on the ATP/ADP ratio of the cell may feedback regulate these same isoforms. Elucidation of the regulatory mechanisms underlying Suc and Glc/Glc-P cycles may, therefore, reveal additional complexity in HK contribution to these substrate cycles and, possibly, to glycolysis regulation in plants.

2.7.1.3 Implications of HK sensitivity to ADP in stress responses

Sensitivity of some HK isoforms to inhibition by ADP may impact greatly on the metabolism of plant cells under O_2 deprivation. Seeds, bulky organs such as potato tuber, or roots of flooded plants experience low ATP/ADP ratios as they become hypoxic, i.e. as respiration is limited by low O_2 tensions (Drew, 1997; Geigenberger, 2003). Plant cells under hypoxia are also characterized by a drop of cytoplasmic pH from 7.5 to 6.8, which compromises their survival (Gout *et al.*, 2001). Cytoplasmic acidosis and a low ATP/ADP

ratio have been claimed to diminish activities of some HK isoforms in hypoxic tissues (Renz and Stitt, 1993; Galina *et al.*, 1995; Bouny and Saglio, 1996; Claeyssen *et al.*, 2006). Thus, inhibition of putative plasma membrane-anchored HK may impede hexose import from the extracellular space under hypoxic stress (Claeyssen *et al.*, 2006). Also, production of G6P for energy-costly pathways may be shut down due to ADP inhibition of mitochondrial HK in hypoxic maize root tips (Galina and da-Silva, 2000). Such impairments may provide a basis for the identification of HK as a major limiting step of glycolysis in maize root tips deprived of O_2 (Bouny and Saglio, 1996). The maintenance of glycolytic flux in acclimated root tips, a requirement for their survival under hypoxia (Xia *et al.*, 1995), was attributed to induced HK activity and its reduced inhibition due to higher cytoplasmic pH (Bouny and Saglio, 1996). Similarly, the flood tolerance of some species of the *Echinochloa* genus has been correlated to specific HK isoforms with acidic pH optima under hypoxia (Fox *et al.*, 1998). These results suggest a major part for some HK isoforms in survival of plant tissues under low O_2 environments. A good candidate may be cytosolic, ADP-insensitive HK, which has been proposed to transiently generate ATP and Glc from ADP and G6P in hypoxic maize root tips (Galina *et al.*, 1995). However, this process remains to be demonstrated. Nonetheless, these data on HK activities agree with those from HK gene expression patterns in that HK appears to play a primordial role in the response of plant cells to hypoxic stress. The above findings suggest that HK has evolved to multiple isoforms with specific subcellular localizations and kinetic properties that serve their individual roles in plant metabolism, under normal and stress conditions.

2.7.2 Function in hexose sensing and signaling

2.7.2.1 HK is a hexose sensor: evidence and mode of action

Beyond its function in hexose metabolism, HK acts as a hexose sensor in plants (Rolland *et al.*, 2006), thus exhibiting a feature common to higher and lower Eukaryotes (Rolland *et al.*, 2001). Sugars serve as nutrients and structural components, but also as signaling molecules that control metabolism and growth and development throughout the plant life cycle (Rolland *et al.*, 2006). At the molecular level, mutant screens have revealed

extensive connections between sugar and hormone signaling pathways (Leon and Sheen, 2003; Rolland *et al.*, 2006). Sugars also activate or repress genes involved in cell cycle regulation, photosynthesis, carbon and nitrogen metabolism, stress responses, germination, vegetative and reproductive development, and senescence (Koch, 1996; Rolland *et al.*, 2002). HK was first implicated as a hexose sensor in mediating repression of photosynthetic and glyoxylate cycle genes by Glc, Fru, Man and Gal (Graham *et al.*, 1994; Jang and Sheen, 1994). Glc analogs and the HK inhibitor mannoheptulose have been used to show that HK mediated gene repression by hexoses, and the arrest of seed germination by Man (Graham *et al.*, 1994; Jang and Sheen, 1994; Pego *et al.*, 1999). Intermediates of downstream Glc metabolism such as P_i or ATP were ineffective in triggering the signal (Jang and Sheen, 1994; Pego *et al.*, 1999). More direct evidence for a role of AtHXK1 and AtHXK2 in Glc sensing was provided with the characterization of transgenic *Arabidopsis* plants with altered HK levels (Jang *et al.*, 1997). Plants overexpressing AtHXK1 or AtHXK2 exhibited Glc hypersensitivity whereas antisense plants were hyposensitive, based on seedling development and marker gene expression. Interestingly, heterologous expression of the yeast Glc sensor HK PII elevated the catalytic activity but reduced Glc sensitivity in the transgenics, suggesting distinct sensing mechanisms in yeast and plants (Jang *et al.*, 1997).

Compelling evidence that HK indeed fulfills two separate functions in *Arabidopsis* came with the characterization of two *Glc insensitive 2* (*gin2*) mutants carrying a mutation in the *AtHXK1* gene (Moore *et al.*, 2003). One *gin2* mutant displayed broad growth defects that became more pronounced with increased light intensity, i.e. in physiological conditions where Glc signaling was enhanced (Moore *et al.*, 2003). Furthermore, the phosphorylating activity of AtHXK1 was uncoupled from its sensing function through point mutations in its two catalytic domains. The engineered proteins displayed no phosphorylating activity, yet they still mediated Glc responses in gene expression, growth and senescence. Also, these constructs restored growth and gene expression to wild-type levels when expressed in *gin2* plants (Moore *et al.*, 2003). Therefore, it was unambiguously demonstrated that Glc sensing and signaling in plants required AtHXK1 but not hexose-Ps or other downstream metabolic products. Moreover, Glc sensing by AtHXK1 was not dependent on its catalytic activity

per se, thus proving that HK exhibits two distinct functions in hexose metabolism and sensing in plants (Moore *et al.*, 2003). Lastly, the AtHXK1-dependent Glc signaling was shown to interact with the auxin and cytokinin signaling pathways. Therefore, HK is a Glc sensor that integrates environmental, nutritional and hormonal cues in the signaling network that governs growth and development (Moore *et al.*, 2003). The broad spectrum of Glc responses affected in *gin2* mutants, including gene expression, cell proliferation, vegetative and reproductive development, reproduction, and senescence, has confirmed the central role of HK-dependent signaling in plant life (Moore *et al.*, 2003). However, much remains to be discovered about the molecular mechanisms underlying hexose sensing and signaling by HK. First, how HK transmits the signal is unclear. It has been suggested that HK may undergo a change in conformation or oligomeric state upon Glc binding, thereby triggering a signal transduction cascade (Frömmer *et al.*, 2003; Moore, 2004). Secondly, it is not known whether the presence of AtHXK1 in both mitochondrial and nuclear fractions is significant to its signaling function. Membrane localization of AtHXK1 has been suggested to be key for its signaling function (Xiao *et al.*, 2000). In maize, inhibition of mitochondrial HK by ADP, mannoheptulose and glucosamine compared to insensitive cytosolic HK has been taken as evidence for the role of mitochondrial HK in hexose sensing (da-Silva *et al.*, 2001). However, this interesting hypothesis awaits more direct demonstration. Glc has been shown to enhance proteasome-dependent degradation of the transcription factor EIN3 in the nucleus through AtHXK1 signaling (Yanagisawa *et al.*, 2003). This suggests that at least the nuclear localization is important for HK-dependent signaling. Other transcription factors have been implicated in Glc signaling (Rolland *et al.*, 2006), which may constitute additional targets for AtHXK1. In these regards, identification and characterization of the partner molecules that interact with AtHXK1 in the nucleus (Fig. 2.1) will provide useful clues on its regulatory interactions (Rolland *et al.*, 2006). These investigations may also clarify whether AtHXK1 is translocated from the mitochondrion to the nucleus, or mediates distinct signaling events depending on its subcellular distribution.

2.7.2.2 Importance of HK in the hexose signaling network

Additional sugar signaling pathways have been unraveled that do not rely on the signaling function of HK. Thus, glycolytic-dependent hexose signaling has been found that involved the catalytic activity of HK and downstream hexose metabolism (Xiao *et al.*, 2000; Lejay *et al.*, 2003). The metabolic role of HK in hexose sensing may help explain the recurrent idea that sugar fluxes into metabolism are more important than their steady-state levels to initiate a response (Koch, 1996; Lalonde *et al.*, 1999; Smeekens, 2000). Furthermore, hexose and Suc responses have been shown to trigger specific signaling pathways that do not involve HK (Chiou and Bush, 1998; Smeekens, 2000; Xiao *et al.*, 2000; Tiessen *et al.*, 2003). HK-independent Glc sensing and signaling may involve cell-surface receptors as extracellular sensors, such as Regulator of G-protein signaling 1 (RGS1) (Chen and Jones, 2004). Targets and processes downstream of RGS1 await further elucidation (Chen and Jones, 2004). HK-independent signaling of Suc has been shown to involve SnRK1 (Tiessen *et al.*, 2003; Halford *et al.*, 2004), which may interact with T6P at least in some cases (Schluepmann *et al.*, 2004; Kolbe *et al.*, 2005). It should be added that FK and GalK have been proposed as additional intracellular sugar sensors besides HK (Pego and Smeekens, 2000; Rolland *et al.*, 2002; Sherson *et al.*, 2003). In particular, FK has been hypothesized to regulate HK activity, by analogy with the mammalian model (Pego and Smeekens, 2000). These considerations reveal a complex interplay of various sugar sensors and signal transduction pathways, and raise questions about their coordination with HK-dependent signaling. In that perspective, sensitivity of SnRK1 activity to inhibition by G6P or F6P (Toroser *et al.*, 2000) suggests possibilities of cross-talk between glycolysis-dependent and HK-independent signaling pathways. Also, the question is open as to which HK isoforms may be involved in HK- versus glycolysis-dependent signaling, and whether their localization or kinetic properties may play a role in one pathway or the other.

2.8 Conclusions and perspectives

It is now well established that plant HK fulfills a catalytic function and another in hexose sensing and signaling. The catalytic function of HK occupies a central place in primary metabolism by providing hexose-Ps to glycolysis and other major pathways in several cell compartments (Fig. 2.1). The level of control of HK on primary metabolism still awaits quantification and hence, determining the flux control coefficient of HK over these pathways will mark a major progress. Of equal importance is the function of HK in hexose sensing and signaling as it integrates environmental and intrinsic cues in the signaling network that controls growth and development (Moore *et al.*, 2003; Rolland *et al.*, 2006). In addition, there may be much left to discover about plant HK if the implication of mammalian HK in apoptosis (Kim and Dang, 2005) proves to be conserved in plants. This may well be the case as mitochondrion-associated NbHXK1 has recently been involved in the control of apoptosis in *Nicotiana benthamiana* cells (Kim *et al.*, 2006). The main future challenge will be to clarify how individual HK isoforms of a plant cell contribute to each function. We are only beginning to understand how the kinetic and regulatory properties of HK isoforms, and their subcellular localizations, may suit them to specific roles. The implication of HK in glycolysis-dependent hexose signaling suggests that a given HK isoform may contribute to hexose metabolism and/or signaling, depending on environmental conditions and developmental stage.

2.9 Acknowledgements

This work was supported by a Discovery Grant from the National Science and Engineering Research Council of Canada to J.R. We are grateful to Drs. Madoka Gray-Mitsumune (IRBV, Université de Montréal) and Nicholas J. Provart (University of Toronto) for their helpful assistance in the use of on-line tools and resources at the BAR and SIGnAL websites.

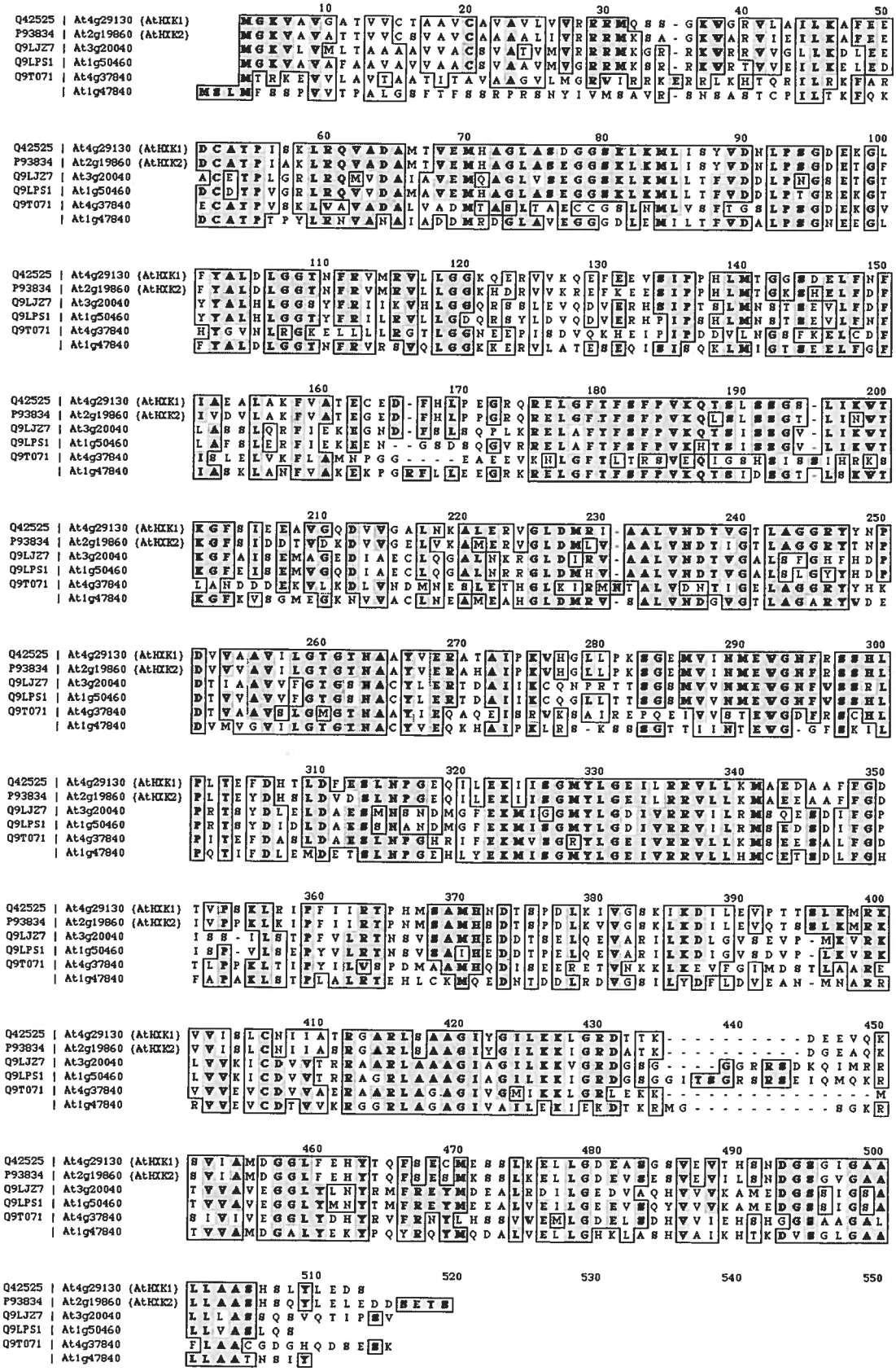
2.10 Supplementary (web-only) data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.12.001.

Supplementary Table 2.1: Names, abbreviations and EC numbers of cited enzymes.

Enzyme name	Abbreviation	EC number
α-amylase		EC 3.2.1.1
β-amylase	BAM	EC 3.2.1.2
α-galactosidase		EC 3.2.1.22
α-glucosidase		EC 3.2.1.20
acid phosphatase		EC 3.1.3.2
ATP-dependent phosphofructokinase	PFK	EC 2.7.1.11
2-deoxy-D-glucose-6-phosphatase		EC 3.1.3.68
fructokinase	FK	EC 2.7.1.4
galactokinase	GalK	EC 2.7.1.6
glucokinase	GK	EC 2.7.1.2
GDP-D-mannose pyrophosphorylase	GMPase	EC 2.7.7.13
D-glucose-1-phosphatase		EC 3.1.3.10
D-glucose-6-phosphatase		EC 3.1.3.9
D-glucose-6-phosphate dehydrogenase	G6PDH	EC 1.1.1.49
hexokinase	HK	EC 2.7.1.1
invertase	INV	EC 3.2.1.26
mannitol dehydrogenase	MTD	EC 1.1.1.255
mannokinase	MK	EC 2.7.1.7
nitrate reductase	NR	EC 1.7.1.1
phospho*enol*pyruvate carboxylase	PEPC	EC 4.1.1.31
phosphoglucomutase	PGM	EC 5.4.2.2

phosphoglucose isomerase	PGI	EC 5.3.1.9
phosphomannose isomerase	PMI	EC 5.3.1.8
phosphomannomutase	PMM	EC 5.4.2.8
D-sucrose-phosphate phosphatase		EC 3.1.3.24
D-sucrose phosphorylase		EC 2.4.1.7
D-sucrose-phosphate synthase	SPS	EC 2.4.1.14
D-sucrose-D-sucrose fructosyltransferase	SST	EC 2.4.1.99
D-sucrose synthase	SuSy	EC 2.4.1.13
transglucosidase (cytosolic or plastidic)	TGD	EC 2.4.1.25
UDP-D-glucose pyrophosphorylase	UGPase	EC 2.7.7.9
D-xylose isomerase		EC 5.3.1.5


Q42525 | At4g29130 (AtHXK1)
P93834 | At2g19860 (AtHXK2)
Q9LJZ7 | At3g20040
Q9LPS1 | At1g50460
Q9T071 | At4g37840
| At1g47840

Supplementary Figure 2.1: Multiple alignment of the predicted HK amino acid sequences of *Arabidopsis thaliana*, using CLUSTAL W (1.82).

UniProt entries of HKs are indicated on the left, followed by their AGI numbers. The trivial names AtHXK1 and AtHXK2 were taken from Jang *et al.* (1997). Identical (dark grey) and similar (light grey) residues shared by *Arabidopsis* HKs are shaded. Gaps are represented by dashes.

Supplementary Table 2.2: Growth stages presented in Fig. 2.2 are from Boyes *et al.* (2001).

Stage number	Approximate number of days after sowing	Description
1		rosette growth
1.02	10.3 (on plates) 12.5	2 rosette leaves > 1 mm in length
1.03	14.4 (on plates) 15.9	3 rosette leaves > 1 mm in length
1.04	16.5	4 rosette leaves > 1 mm in length
1.09	21.1	9 rosette leaves > 1 mm in length
1.1	21.6	10 rosette leaves > 1 mm in length
1.11	22.2	11 rosette leaves > 1 mm in length
1.12	23.31	12 rosette leaves > 1 mm in length
3.7	27.4	rosette is 70% of final size
6.1	35.9	10% flowers to be produced are open
6.5	43.5	50% flowers to be produced are open
9.70		senescence complete

2.11 References

Alonso, A.P., Vigeolas, H., Raymond, P., Rolin, D., Dieuaide-Noubhani, M., 2005. A new substrate cycle in plants. Evidence for a high glucose-phosphate-to-glucose turnover from in vivo steady-state and pulse-labeling experiments with [^{13}C]glucose and [^{14}C]glucose. Plant Physiol. 138, 2220-2232.

Appeldoorn, N.J., Sergeeva, L., Vreugdenhil, D., Van Der Plas, L.H., Visser, R.G., 2002. *In situ* analysis of enzymes involved in sucrose to hexose-phosphate conversion during stolon-to-tuber transition of potato. Physiol. Plant. 115, 303-310.

Axelrod, B., Bandurski, R.S., Grenier, C.M., Jang, R., 1953. The metabolism of hexose and pentose phosphates in higher plants. J. Biol. Chem. 202, 619-634.

Balasubramanian, R., Karve, A., Kandasamy, M., Meagher, R., Moore, B., 2005. A role for the actin cytoskeleton in hexokinase mediated glucose signaling. XVIth International Conference on *Arabidopsis* Research. TAIR publication 501716579 (Abstract).

Baldus, B., Kelly, G.J., Latzko, E., 1981. Hexokinases of spinach leaves. Phytochem. 20, 1811-1814.

Baroja-Fernandez, E., Munoz, F.J., Saikusa, T., Rodriguez-Lopez, M., Akazawa, T., Pozueta-Romero, J., 2003. Sucrose synthase catalyzes the *de novo* production of ADPglucose linked to starch biosynthesis in heterotrophic tissues of plants. Plant Cell Physiol. 44, 500-509.

Baud, S., Boutin, J.P., Miquel, M., Lepiniec, L., Rochat, C., 2002. An integrated overview of seed development in *Arabidopsis thaliana* ecotype WS. Plant Physiol. Biochem. 40, 151-160.

Baud, S., Graham, I.A., 2006. A spatiotemporal analysis of enzymatic activities associated with carbon metabolism in wild-type and mutant embryos of Arabidopsis using *in situ* histochemistry. Plant J. 46, 155-169.

Beevers, H., Gibbs, M., 1954. Position of C14 in alcohol and carbon dioxide formed from labeled glucose by corn root tips. Plant Physiol. 29, 318-321.

Benning, C., Ohta, H., 2005. Three enzyme systems for galactoglycerolipid biosynthesis are coordinately regulated in plants. J. Biol. Chem. 280, 2397-2400.

Benning, C., Xu, C., Awai, K., 2006. Non-vesicular and vesicular lipid trafficking involving plastids. Curr. Opin. Plant Biol. 9, 241-247.

Borchert, S., Harborth, J., Schunemann, D., Hoferichter, P., Heldt, H.W., 1993. Studies of the enzymatic capacities and transport properties of pea root plastids. Plant Physiol. 101, 303-312.

Bouny, J.M., Saglio, P.H., 1996. Glycolytic flux and hexokinase activities in anoxic maize root tips acclimated by hypoxic pretreatment. Plant Physiol. 111, 187-194.

Bowles, D., Lim, E.K., Poppenberger, B., Vaistij, F.E., 2006. Glycosyltransferases of lipophilic small molecules. Annu. Rev. Plant Biol. 57, 567-597.

Boyes, D.C., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., Gorlach, J., 2001. Growth stage-based phenotypic analysis of *Arabidopsis*: a model for high throughput functional genomics in plants. Plant Cell 13, 1499-1510.

Bozzo, G.G., Raghothama, K.G., Plaxton, W.C., 2004. Structural and kinetic properties of a novel purple acid phosphatase from phosphate-starved tomato (*Lycopersicon esculentum*) cell cultures. Biochem. J. 377, 419-428.

Cárdenas, M.L., Cornish-Bowden, A., Ureta,T., 1998. Evolution and regulatory role of the hexokinases. Biochim. Biophys. Acta 1401, 242-264.

Cernac, A., Benning, C., 2004. WRINKLED1 encodes an AP2/EREB domain protein involved in the control of storage compound biosynthesis in *Arabidopsis*. Plant J. 40, 575-585.

Chabregas, S.M., Luche, D.D., Farias, L.P., Ribeiro, A.F., van Sluys, M.A., Menck, C.F., Silva-Filho, M.C., 2001. Dual targeting properties of the N-terminal signal sequence of *Arabidopsis thaliana* THI1 protein to mitochondria and chloroplasts. Plant Mol. Biol. 46, 639-650.

Chen, J.G., Jones, A.M., 2004. AtRGS1 function in *Arabidopsis thaliana*. Meth. Enzymol. 389, 338-350.

Chew, O., Rudhe, C., Glaser, E., Whelan, J., 2003. Characterization of the targeting signal of dual-targeted pea glutathione reductase. Plant Mol. Biol. 53, 341-356.

Chia, T., Thorneycroft, D., Chapple, A., Messerli, G., Chen, J., Zeeman, S.C., Smith, S.M., Smith, A.M., 2004. A cytosolic glucosyltransferase is required for conversion of starch to sucrose in *Arabidopsis* leaves at night. Plant J. 37, 853-863.

Chiou, T.J. Bush, D.R., 1998. Sucrose is a signal molecule in assimilate partitioning. Proc. Natl. Acad. Sci. USA 95, 4784-4788.

Cho, J.I., Ryoo, N., Ko, S., Lee, S.K., Lee, J., Jung, K.H., Lee, Y.H., Bhoo, S.H., Winderickx, J., An, G., Hahn, T.R., Jeon, J.S., 2006. Structure, expression, and functional analysis of the hexokinase gene family in rice (*Oryza sativa* L.). Planta 224, 598-611.

Claeyssen, E., Wally, O., Matton, D.P., Morse, D., Rivoal, J., 2006. Cloning, expression, purification, and properties of a putative plasma membrane hexokinase from *Solanum chacoense*. Protein Expr. Purif. 47, 329-339.

Cleary, S.P., Tan, F.C., Nakrieko, K.A., Thompson, S.J., Mullineaux, P.M., Creissen, G.P., von Stedingk, E., Glaser, E., Smith, A.G., Robinson, C., 2002. Isolated plant mitochondria import chloroplast precursor proteins in vitro with the same efficiency as chloroplasts. J. Biol. Chem. 277, 5562-5569.

Clément, C., Chavant, L., Burrus, M., Audran, J.C., 1994. Anther starch variations in *lilium* during pollen development. Sex. Plant Reprod. 7, 347-356.

Collings, D.A., Carter, C.N., Rink, J.C., Scott, A.C., Wyatt, S.E., Allen, N.S., 2000. Plant nuclei can contain extensive grooves and invaginations. Plant Cell 12, 2425-2440.

Colowick, S.P., Kalckar, H.M., 1943. The role of myokinase in transphosphorylations. I. The enzymatic phosphorylation of hexoses by adenyl pyrophosphate. J. Biol. Chem. 148, 117-126.

Conklin, P.L., 2001. Recent advances in the role and biosynthesis of ascorbic acid in plants. Plant Cell Environ. 24, 383-394.

Cook, D., Fowler, S., Fiehn, O., Thomashow, M.F., 2004. A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. Proc. Natl. Acad. Sci. USA 101, 15243-15248.

Critchley, J.H., Zeeman, S.C., Takaha, T., Smith, A.M., Smith, S.M., 2001. A critical role for disproportionating enzyme in starch breakdown is revealed by a knock-out mutation in *Arabidopsis*. Plant J. 26, 89-100.

da-Silva, W.S., Rezende, G.L., Galina, A., 2001. Subcellular distribution and kinetic properties of cytosolic and non-cytosolic hexokinases in maize seedling roots: implications for hexose phosphorylation. J. Exp. Bot. 52, 1191-1201.

Dai, N., Kandel-Kfir, M., Petreikov, M., Hanael, R., Levin, I., Ricard, B., Rothan, C., Schaffer, A.A., Granot, D., 2002. The tomato hexokinase LeHXK1 cloning,

mapping, expression pattern and phylogenetic relationships. Plant Sci. 163, 581-590.

Dai, N., Schaffer, A., Petreikov, M., Shahak, Y., Giller, Y., Ratner, K., Levine, A., Granot, D., 1999. Overexpression of Arabidopsis hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. Plant Cell 11, 1253-1266.

Damari-Weissler, H., Kandel-Kfir, M., Gidoni, D., Mett,A., Belausov,E., Granot,D., 2006. Evidence for intracellular spatial separation of hexokinases and fructokinases in tomato plants. Planta 224, 1495-1502.

Davies, H.V., Shepherd, L.V., Burrell, M.M., Carrari, F., Urbanczyk-Wochniak, E., Leisse, A., Hancock, R.D., Taylor, M., Viola, R., Ross, H., McRae, D., Willmitzer, L., Fernie, A.R., 2005. Modulation of fructokinase activity of potato (*Solanum tuberosum*) results in substantial shifts in tuber metabolism. Plant Cell Physiol. 46, 1103-1115.

Davis,J.M., Loescher, W.H., 1990. [^{14}C]-Assimilate translocation in the light and dark in celery (*Apium graveolens*) leaves of different ages. Physiol. Plant. 79, 656-662.

Dey, P.M., 1983. Galactokinase of *Vicia faba* seeds. Eur. J. Biochem. 136, 155-159.

Dieuaide-Noubhani, M., Raffard, G., Canioni, P., Pradet, A., Raymond, P., 1995. Quantification of compartmented metabolic fluxes in maize root tips using isotope distribution from ^{13}C- or ^{14}C-labeled glucose. J. Biol. Chem. 270, 13147-13159.

Doehlert, D.C., 1989. Separation and characterization of four hexose kinases from developing maize kernels. Plant Physiol. 89, 1042-1048.

Drew, M.C., 1997. Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 223-250.

Dry, I.B., Nash, D., Wiskich, J.T., 1983. The mitochondrial localization of hexokinase in pea leaves. Planta 158, 152-156.

Duchêne, A.M., Giritch, A., Hoffmann, B., Cognat, V., Lancelin, D., Peeters, N.M., Zaepfel, M., Maréchal-Drouard,L., Small, I.D., 2005. Dual targeting is the rule for organellar aminoacyl-tRNA synthetases in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 102, 16484-16489.

Duff, S.M.G., Lefebvre, D.D., Plaxton, W.C., 1989. Purification and characterization of a phospho*enol*pyruvate phosphatase from *Brassica nigra* suspension cell cultures. Plant Physiol. 90, 734-741.

Eastmond, P.J., Rawsthorne, S., 2000. Coordinate changes in carbon partitioning and plastidial metabolism during the development of oilseed rape embryos. Plant Physiol. 122, 767-774.

Eastmond, P.J., van Dijken, A.J., Spielman, M., Kerr, A., Tissier, A.F., Dickinson, H.G., Jones, J.D., Smeekens, S.C., Graham, I.A., 2002. Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. Plant J. 29, 225-235.

Emanuelsson, O., Nielsen, H., Brunak, S., von Heijne, G., 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300, 1005-1016.

Etxeberria, E., Baroja-Fernandez, E., Munoz, F.J., Pozueta-Romero, J., 2005a. Sucrose-inducible endocytosis as a mechanism for nutrient uptake in heterotrophic plant cells. Plant Cell Physiol. 46, 474-481.

Etxeberria, E., Gonzalez, P., Tomlinson, P., Pozueta-Romero, J., 2005b. Existence of two parallel mechanisms for glucose uptake in heterotrophic plant cells. J. Exp. Bot. 56, 1905-1912.

Farré, E.M., Tiessen, A., Roessner, U., Geigenberger, P., Trethewey, R.N., Willmitzer, L., 2001. Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. Plant Physiol. 127, 685-700.

Fernie, A.R., Tiessen, A., Stitt, M., Willmitzer, L., Geigenberger, P., 2002a. Altered metabolic fluxes result from shifts in metabolite levels in sucrose phosphorylase-expressing potato tubers. Plant, Cell Environ. 25, 1219-1232.

Fernie, A.R., Willmitzer, L., Trethewey, R.N., 2002b. Sucrose to starch: a transition in molecular plant physiology. Trends Plant Sci. 7, 35-41.

Focks, N., Benning, C., 1998. wrinkled1: a novel, low-seed-oil mutant of *Arabidopsis* with a deficiency in the seed-specific regulation of carbohydrate metabolism. Plant Physiol. 118, 91-101.

Fox, T.C., Green, B.J., Kennedy, R.A., Rumpho, M.E., 1998. Changes in hexokinase activity in *Echinochloa phyllopogon* and *Echinochloa crus-pavonis* in response to abiotic stress. Plant Physiol. 118, 1403-1409.

Frömmer, W.B., Schulze, W.X., Lalonde, S., 2003. Hexokinase, Jack-of-all-trades. Science 300, 261-263.

Fujiki, Y., Yoshikawa, Y., Sato, T., Inada, N., Ito, M., Nishida, I., Watanabe, A., 2001. Dark-inducible genes from *Arabidopsis thaliana* are associated with leaf senescence and repressed by sugars. Physiol Plant. 111, 345-352.

Galina, A., da-Silva, W.S., 2000. Hexokinase activity alters sugar-nucleotide formation in maize root homogenates. Phytochem. 53, 29-37.

Galina, A., Logullo, L., de Sousa, E.F., Rezende, G.L., da-Silva, W.S., 1999. Sugar phosphorylation modulates ADP inhibition of maize mitochondrial hexokinase. Physiol. Plant. 105, 17-23.

Galina, A., Reis, M., Albuquerque, M.C., Puyou, A.G., Puyou, M.T., de Meis, L., 1995. Different properties of the mitochondrial and cytosolic hexokinases in maize roots. Biochem. J. 309, 105-112.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D., Bairoch, A., 2003. ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 31, 3784-3788.

Geigenberger, P., 2003. Response of plant metabolism to too little oxygen. Curr. Opin. Plant Biol. 6, 247-256.

Gerhardt, R., Stitt, M., Heldt, H.W., 1987. Subcellular metabolite levels in spinach leaves - regulation of sucrose synthesis during diurnal alterations in photosynthetic partitioning. Plant Physiol. 83, 399-407.

German, M.A., Dai, N., Matsevitz, T., Hanael, R., Petreikov, M., Bernstein, N., Ioffe, M., Shahak, Y., Schaffer, A.A., Granot, D., 2003. Suppression of fructokinase encoded by LeFRK2 in tomato stem inhibits growth and causes wilting of young leaves. Plant J. 34, 837-846.

Giegé, P., Heazlewood, J.L., Ressner-Tunali, U., Millar, H., Fernie, A.R., Leaver, C.J., Sweetlove, L.J., 2003. Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. Plant Cell 15, 2140-2151.

Giese, J.O., Herbers, K., Hoffmann, M., Klosgen, R.B., Sonnewald, U., 2005. Isolation and functional characterization of a novel plastidic hexokinase from *Nicotiana tabacum*. FEBS Lett. 579, 827-831.

Goetz, M., Godt, D.E., Guivarc'h, A., Kahmann, U., Chriqui, D., Roitsch, T., 2001. Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. Proc. Natl. Acad. Sci. USA 98, 6522-6527.

Gomez, L.D., Baud, S., Gilday, A., Li, Y., Graham, I.A., 2006. Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. Plant J. 46, 69-84.

Gout, E., Boisson, A.M., Aubert, S., Douce, R., Bligny, R., 2001. Origin of the cytoplasmic pH changes during anaerobic stress in higher plant cells. carbon-13 and phosphorous-31 Nuclear Magnetic Resonance studies. Plant Physiol. 125, 912-925.

Graham, I.A., Denby, K.J., Leaver, C.J., 1994. Carbon catabolite repression regulates glyoxylate cycle gene expression in cucumber. Plant Cell 6, 761-772.

Halford, N.G., Hey, S., Jhurreea, D., Laurie, S., McKibbin, R.S., Zhang, Y., Paul, M.J., 2004. Highly conserved protein kinases involved in the regulation of carbon and amino acid metabolism. J. Exp. Bot. 55, 35-42.

Haritatos, E., Ayre, B.G., Turgeon, R., 2000. Identification of phloem involved in assimilate loading in leaves by the activity of the galactinol synthase promoter. Plant Physiol. 123, 929-937.

Häusler, R.E., Schlieben, N.H., Schulz, B., Flügge, U.I., 1998. Compensation of decreased triose phosphate phosphate translocator activity by accelerated starch turnover and glucose transport in transgenic tobacco. Planta 204, 366-376.

Higgins, T.J., Easterby, J.S., 1974. Wheatgerm hexokinase: physical and active-site properties. Eur. J. Biochem. 45, 147-160.

Hill, L.M., Morley-Smith, E.R., Rawsthorne, S., 2003. Metabolism of sugars in the endosperm of developing seeds of oilseed rape. Plant Physiol. 131, 228-236.

Hooks, M.A., Shearer, G.C., Roberts, J.K.M., 1994. Nucleotide availability in maize (*Zea mays* L.) root tips (estimation of free and protein-bound nucleotides using ^{31}P-

Nuclear Magnetic Resonance and a novel protein ligand binding assay). Plant Physiol. 104, 581-589.

Huber,S.C., Akazawa, T., 1986. A novel sucrose synthase pathway for sucrose degradation in cultured sycamore cells. Plant Physiol. 81, 1008-1013.

Jang, J.C., Leon, P., Zhou, L., Sheen, J., 1997. Hexokinase as a sugar sensor in higher plants. Plant Cell 9, 5-19.

Jang, J.C., Sheen, J., 1994. Sugar sensing in higher plants. Plant Cell 6, 1665-1679.

Jannaschk, D., Burgos, M., Centerlles, J.J., Ovadi, J., Cascante, M., 1999. Application of metabolic control analysis to the study of toxic effects of copper in muscle glycolysis. FEBS Lett. 445, 144-148

Junker, B.H., Wuttke, R., Nunes-Nesi, A., Steinhauser, D., Schauer, N., Bussis, D., Willmitzer, L., Fernie, A.R., 2006. Enhancing vacuolar sucrose cleavage within the developing potato tuber has only minor effects on metabolism. Plant Cell Physiol 47, 277-289.

Kandel-Kfir, M., Damari-Weissler, H., German, M.A., Gidoni ,D., Mett, A., Belausov, E., Petreikov, M., Adir, N., Granot, D., 2006. Two newly identified membrane-associated and plastidic tomato HXKs: characteristics, predicted structure and intracellular localization. Planta. 224, 1341-1352.

Kaplan, C.P., Tugal, H.B., Baker, A., 1997. Isolation of a cDNA encoding an *Arabidopsis* galactokinase by functional expression in yeast. Plant Mol. Biol. 34, 497-506.

Kashiwaya, Y., Sato, K., Tsuchiya, N., Thomas, S., Fell, D.A., Veech, R.L., Passonneau, J.V., 1994. Control of glucose utilization in working perfused rat heart. J. Biol. Chem. 269, 25502-25514.

Keegstra, K., Raikhel, N., 2001. Plant glycosyltransferases. Curr. Opin. Plant Biol. 4, 219-224.

Keller, F., Pharr, D.M., 1996. Metabolism of carbohydrates in sinks and sources: Galactosyl-Suc oligosaccharides. In: Zamski, E., Schaffer, A. A. (Eds.), *Photoassimilate distribution in plants and crops. Source-sink relationships.* M. Dekker Inc., New York, NY, USA, pp. 157-183.

Kim,J.W., Dang, C.V., 2005. Multifaceted roles of glycolytic enzymes. Trends Biochem. Sci. 30, 142-150.

Kim, M., Lim, J.H., Ahn, C.S., Park, K., Kim, G.T., Kim, W.T., Pai, H.S., 2006. Mitochondria-associated hexokinases play a role in the control of programmed cell death in *Nicotiana benthamiana.* Plant Cell 18, 2341-2355

Kim, J.Y., Mahe, A., Brangeon, J., Prioul, J.L., 2000. A maize vacuolar invertase, IVR2, is induced by water stress. Organ/tissue specificity and diurnal modulation of expression. Plant Physiol 124, 71-84.

Koch, K.E., 1996. Carbohydrate-molulated gene expression in plants. Annu. Rev. Plant Physiol Plant Mol. Biol. 47, 509-540.

Koch, K.E., 2004. Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr. Opin. Plant Biol. 7, 235-246.

Köhler, R.H., Cao, J., Zipfel, W.R., Webb, W.W., Hanson, M.R., 1997. Exchange of protein molecules through connections between higher plant plastids. Science 276, 2039-2042.

Kolbe, A., Tiessen, A., Schluepmann, H., Paul, M., Ulrich, S., Geigenberger, P., 2005. Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. Proc. Natl. Acad. Sci. USA 102, 11118-11123.

Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X., Harper, J.F., 2002. Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. Plant Physiol. 130, 2129-2141.

Kruger, N.J., von Schaewen, A., 2003. The oxidative pentose phosphate pathway: structure and organisation. Curr. Opin. Plant Biol. 6, 236-246.

Kwok, E.Y., Hanson, M.R., 2004. Plastids and stromules interact with the nucleus and cell membrane in vascular plants. Plant Cell Rep. 23, 188-195.

Lalonde, S., Boles, E., Hellmann, H., Barker, L., Patrick, J.W., Frömmer, W.B., Ward, J.M., 1999. The dual function of sugar carriers. Transport and sugar sensing. Plant Cell 11, 707-726.

Lalonde, S., Wipf, D., Frömmer, W.B., 2004. Transport mechanisms for organic forms of carbon and nitrogen between source and sink. Annu. Rev. Plant Biol. 55, 341-372.

Lee, D.C., Cottrill, M.A., Forsberg, C.W., Jia, Z., 2003. Functional insights revealed by the crystal structures of *Escherichia coli* glucose-1-phosphatase. J. Biol. Chem. 278, 31412-31418.

Leidreiter, K., Kruse, A., Heineke, D., Robinson, D.G., Heldt, H.W., 1995. Subcellular volumes and metabolite concentrations in potato (*Solanum tuberosum* Cv Desiree) leaves. Bot. Acta 108, 439-444.

Lejay, L., Gansel, X., Cerezo, M., Tillard, P., Muller, C., Krapp, A., von Wiren, N., Daniel-Vedele, F., Gojon, A., 2003. Regulation of root ion transporters by photosynthesis: functional importance and relation with hexokinase. Plant Cell 15, 2218-2232.

Leon, P., Sheen, J., 2003. Sugar and hormone connections. Trends Plant Sci. 8, 110-116.

Levitan, A., Trebitsh, T., Kiss, V., Pereg, Y., Dangoor, I., Danon, A., 2005. Dual targeting of the protein disulfide isomerase RB60 to the chloroplast and the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA 102, 6225-6230.

Liu, F., Vantoai, T., Moy, L.P., Bock, G., Linford, L.D., Quackenbush, J., 2005. Global transcription profiling reveals comprehensive insights into hypoxic response in Arabidopsis. Plant Physiol. 137, 1115-1129.

Loescher, W.H., Tyson, R.H., Everard, J.D., Redgwell, R.J., Bieleski, R.L., 1992. Mannitol synthesis in higher plants: Evidence for the role and characterization of a NADPH-dependent mannose 6-phosphate reductase. Plant Physiol. 98, 1396-1402.

Loreti, E., Poggi, A., Novi, G., Alpi, A., Perata, P., 2005. A genome-wide analysis of the effects of sucrose on gene expression in Arabidopsis seedlings under anoxia. Plant Physiol. 137, 1130-1138.

Lu, Y., Sharkey, T.D., 2004. The role of amylomaltase in maltose metabolism in the cytosol of photosynthetic cells. Planta 218, 466-473.

Martinez-Barajas, E., Randall, D.D., 1998. Purification and characterization of a glucokinase from young tomato (*Lycopersicon esculentum* L. Mill.) fruit. Planta 205, 567-573.

Menu, T., Rothan, C., Dai, N., Petreikov, M., Etienne, C., Destrac-Irvine, A., Schaffer, A., Granot, D., Ricard, B., 2001. Cloning and characterization of a cDNA encoding hexokinase from tomato. Plant Sci. 160, 209-218.

Menu, T., Saglio, P., Granot, D., Dai, N., Raymond, P., Ricard, B., 2004. High hexokinase activity in tomato fruit perturbs carbon and energy metabolism and reduces fruit and seed size. Plant Cell Environ. 27, 89-98.

Meyerhof, O., 1927. Über die enzymatische Milchsäurebildung im Muskelextrakt. III. Mitteilung: Die Milchäurebildung aus den gärfähigen Hexosen. Biochem. Z. 183, 176-215.

Meyerhof, O., 1935. Über die Wirkungsweise der Hexokinase. Naturwissenschaften 23, 850-851

Miernyk, J.A., Dennis, D.T., 1983. Mitochondrial, plastid, and cytosolic isozymes of hexokinase from developing endosperm of *Ricinus communis*. Arch. Biochem. Biophys. 226, 458-468.

Millerd, A., Bonner, J., Axelrod, B., Bandurski, R., 1951. Oxidative and phosphorylative activity of plant mitochondria. Proc. Natl. Acad. Sci. USA 37, 855-862.

Mithieux, G., Rajas, F., Gautier-Stein, A., 2004. A novel role for glucose 6-phosphatase in the small intestine in the control of glucose homeostasis. J. Biol. Chem. 279, 44231-44234.

Moore, B., 2004. Bifunctional and moonlighting enzymes: lighting the way to regulatory control. Trends Plant Sci. 9, 221-228.

Moore, B., Palmquist, D.E., Seemann, J.R., 1997. Influence of plant growth at high CO_2 concentrations on leaf content of ribulose-1,5-bisphosphate carboxylase/oxygenase and intracellular distribution of soluble carbohydrates in tobacco, snapdragon, and parsley. Plant Physiol. 115, 241-248.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Liu, Y.X., Hwang, I., Jones, T., Sheen, J., 2003. Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science 300, 332-336.

Murata, T., Katagiri, H., Ishihara, H., Shibasaki, Y., Asano, T., Toyoda, Y., Pekiner, B., Pekiner, C., Miwa, I., Oka, Y., 1997. Co-localization of glucokinase with actin filaments. FEBS Lett. 406, 109-113.

Nassoury, N., Morse, D., 2005. Protein targeting to the chloroplasts of photosynthetic eukaryotes: getting there is half the fun. Biochim. Biophys. Acta 1743, 5-19.

Neuhaus, H.E., Emes, M.J., 2000. Nonphotosynthetic metabolism in plastids. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 111-140.

Neuhaus, H.E., Thom, E., Mohlmann, T., Steup, M., Kampfenkel, K., 1997. Characterization of a novel eukaryotic ATP/ADP translocator located in the plastid envelope of *Arabidopsis thaliana* L. Plant J. 11, 73-82.

Nguyen-Quoc, B., Foyer, C.H., 2001. A role for 'futile cycles' involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. J. Exp. Bot. 52, 881-889.

Niittylä, T., Messerli, G., Trevisan, M., Chen, J., Smith, A.M., Zeeman, S.C., 2004. A previously unknown maltose transporter essential for starch degradation in leaves. Science 303, 87-89.

Odanaka, S., Bennett, A.B., Kanayama, Y., 2002. Distinct physiological roles of fructokinase isozymes revealed by gene-specific suppression of Frk1 and Frk2 expression in tomato. Plant Physiol. 129, 1119-1126.

Olsson, T., Thelander, M., Ronne, H., 2003. A novel type of chloroplast stromal hexokinase is the major glucose-phosphorylating enzyme in the moss Physcomitrella patens. J. Biol. Chem. 278, 44439-44447.

Paul, M., Pellny, T., Goddijn, O., 2001. Enhancing photosynthesis with sugar signals. Trends Plant Sci. 6, 197-200.

Pedley, K.C., Jones, G.E., Magnani, M., Rist, R.J., Naftalin, R.J., 1993. Direct observation of hexokinase translocation in stimulated macrophages. Biochem. J. 291, 515-522.

Peeters, N., Small, I., 2001. Dual targeting to mitochondria and chloroplasts. Biochim. Biophys. Acta 1541, 54-63.

Pego, J.V., Smeekens, S.C., 2000. Plant fructokinases: a sweet family get-together. Trends Plant Sci. 5, 531-536.

Pego, J.V., Weisbeek, P.J., Smeekens, S.C., 1999. Mannose inhibits *Arabidopsis* germination via a hexokinase-mediated step. Plant Physiol. 119, 1017-1023.

Plaxton, W.C., 1996. The organization and regulation of plant glycolysis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 185-214.

Preiss, J., Sivak, M.N., 1996. Starch synthesis in sinks and sources. In: Zamski, E., Schaffer, A.A. (Eds.), *Photoassimilate Distribution in Plants and Crops. Source-Sink Relationships.* M. Dekker Inc., New York, NY, USA, pp. 63-96.

Provart, N.J., Gil, P., Chen, W., Han, B., Chang, H.S., Wang, X., Zhu, T., 2003. Gene expression phenotypes of *Arabidopsis* associated with sensitivity to low temperatures. Plant Physiol. 132, 893-906.

Puigjaner, J., Rais, B., Burgos, M., Comin, B., Ovadi, J., Cascante, M., 1997. Comparison of control analysis data using different approaches: modelling and experiments with muscle extract. FEBS Lett. 418, 47-52.

Quick, W.P., Schaffer, A.A., 1996. Sucrose metabolism in sources and sinks. In: Zamski, E., Schaffer, A.A. (Eds.), *Photoassimilate distribution in plants and crops. Source-sink relationships.* M. Dekker Inc., New York, NY, USA, pp. 115-156.

Rapoport, T.A., Heinrich, R., Jacobasch, G., Rapoport, S., 1974. A linear steady-state treatment of enzymatic chains. A mathematical model of glycolysis of human erythrocytes. Eur. J. Biochem. 42, 107-120

Renz, A., Merlo, L., Stitt, M., 1993. Partial purification from potato tubers of three fructokinases and three hexokinases which show differing organ and developmental specificity. Planta 190, 156-165.

Renz, A., Stitt, M., 1993. Substrate specificity and product inhibition of different forms of fructokinases and hexokinases in developing potato tubers. Planta 190, 166-175.

Ritsema, T., Smeekens, S.C., 2003. Engineering fructan metabolism in plants. J Plant Physiol. 160, 811-820.

Ritte, G., Raschke, K., 2003. Metabolite export of isolated guard cell chloroplasts of *Vicia faba*. New Phytol. 159, 195-202.

Roitsch, T., Gonzalez, M.C., 2004. Function and regulation of plant invertases: sweet sensations. Trends Plant Sci. 9, 606-613.

Rolland, F., Baena-Gonzalez, E., Sheen, J., 2006. Sugar sensing and signaling in plants: Conserved and novel mechanisms. Annu. Rev. Plant Biol. 57, 675-709.

Rolland, F., Moore, B., Sheen, J., 2002. Sugar sensing and signaling in plants. Plant Cell 14 (Suppl.), S185-S205.

Rolland, F., Winderickx, J., Thevelein, J.M., 2001. Glucose-sensing mechanisms in eukaryotic cells. Trends Biochem. Sci. 26, 310-317.

Rolletschek, H., Koch, K., Wobus, U., Borisjuk, L., 2005. Positional cues for the starch/lipid balance in maize kernels and resource partitioning to the embryo. Plant J. 42, 69-83.

Rontein, D., Dieuaide-Noubhani, M., Dufourc, E.J., Raymond, P., Rolin, D., 2002. The metabolic architecture of plant cells. Stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. J. Biol. Chem. 277, 43948-43960.

Rumpho, M.E., Edwards, G.E., Loescher, W.H., 1983. A pathway for photosynthetic carbon flow to mannitol in celery leaves - Activity and localization of key enzymes. Plant Physiol. 73, 869-873.

Ruuska, S.A., Girke, T., Benning, C., Ohlrogge, J.B., 2002. Contrapuntal networks of gene expression during *Arabidopsis* seed filling. Plant Cell 14, 1191-1206.

Saltman, P., 1953. Hexokinase in higher plants. J. Biol. Chem. 200, 145-154.

Schluepmann, H., Pellny, T., van Dijken, A., Smeekens, S., Paul, M., 2003. Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA 100, 6849-6854.

Schluepmann, H., van Dijken, A., Aghdasi, M., Wobbes, B., Paul,M., Smeekens, S., 2004. Trehalose mediated growth inhibition of Arabidopsis seedlings is due to trehalose-6-phosphate accumulation. Plant Physiol. 135, 879-890.

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Scholkopf,B., Weigel,D., Lohmann,J.U., 2005. A gene expression map of *Arabidopsis thaliana* development. Nat. Genet. 37, 501-506.

Schnarrenberger, C., 1990. Characterization and compartmentation, in green leaves, of hexokinases with different specificities for glucose, fructose, and mannose and for nucleoside triphosphates. Planta 181, 249-255.

Schwender, J., Ohlrogge, J.B., Shachar-Hill, Y., 2003. A flux model of glycolysis and the oxidative pentosephosphate pathway in developing *Brassica napus* embryos. J. Biol. Chem. 278, 29442-29453.

Seifert, G.J., 2004. Nucleotide sugar interconversions and cell wall biosynthesis: how to bring the inside to the outside. Curr. Opin. Plant Biol. 7, 277-284.

Servaites, J.C., Geiger, D.R., 2002. Kinetic characteristics of chloroplast glucose transport. J. Exp. Bot. 53, 1581-1591.

Sherson, S.M., Alford, H.L., Forbes, S.M., Wallace, G., Smith, S.M., 2003. Roles of cell-wall invertases and monosaccharide transporters in the growth and development of *Arabidopsis*. J. Exp. Bot. 54, 525-531.

Smeekens, S., 2000. Sugar-induced signal transduction in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 49-81.

Smith, A.M., Zeeman, S.C., Smith, S.M., 2005. Starch degradation. Annu. Rev. Plant Biol. 56, 73-98.

Spagnoletta, A., De Santis, A., Palmieri, F., Genchi, G., 2002. Purification and characterization of the reconstitutively active adenine nucleotide carrier from mitochondria of Jerusalem artichoke (*Helianthus tuberosus* L.) tubers. J. Bioenerg. Biomembr. 34, 465-472.

Staehelin, L.A., 1997. The plant ER: a dynamic organelle composed of a large number of discrete functional domains. Plant J. 11, 1151-1165.

Stitt, M., Bulpin, P.V., ap-Rees, T., 1978. Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum*. Biochim. Biophys. Acta 544, 200-214.

Stitt, M., Heldt, H.W., 1981. Physiological rates of starch breakdown in isolated intact spinach chloroplasts. Plant Physiol. 68, 755-761.

Stoop, J.M., Pharr, D.M., 1992. Partial purification and characterization of mannitol: mannose 1-oxidoreductase from celeriac (*Apium graveolens* var. rapaceum) roots. Arch. Biochem. Biophys. 298, 612-619.

Stumpf, P.K., 1952. Glycolytic enzymes in higher plants. Annu. Rev. Plant Physiol. 3, 17-34.

Sturm, A., Tang, G.Q., 1999. The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. Trends Plant Sci. 4, 401-407.

Subbaiah, C.C., Palaniappan, A., Duncan, K., Rhoads, D.M., Huber, S.C., Sachs, M.M., 2006. Mitochondrial localization and putative signaling function of sucrose synthase in maize. J. Biol. Chem. 281, 15625-15635.

Sugden, C., Donaghy, P.G., Halford, N.G., Hardie, D.G., 1999. Two SNF1-related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-

coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase *in vitro*. Plant Physiol. 120, 257-274.

Tanner, G.J., Copeland, L., Turner, J.F., 1983. Subcellular localization of hexose kinases in pea stems: mitochondrial hexokinase. Plant Physiol. 72, 659-663.

The Arabidopsis Initiative, 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408, 796-815.

Thevelein, J.M., Hohmann, S., 1995. Trehalose synthase: guard to the gate of glycolysis in yeast? Trends Biochem. Sci. 20, 3-10.

Thévenot, C., Simond-Coté, E., Reyss, A., Manicacci, D., Trouverie, J., Le Guilloux, M., Ginhoux, V., Sidicina, F., Prioul, J.L., 2005. QTLs for enzyme activities and soluble carbohydrates involved in starch accumulation during grain filling in maize. J. Exp. Bot. 56, 945-958.

Tiessen, A., Prescha, K., Branscheid, A., Palacios, N., McKibbin, R., Halford, N.G., Geigenberger, P., 2003. Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. Plant J. 35, 490-500.

Toroser, D., Plaut, Z., Huber, S.C., 2000. Regulation of a plant SNF1-related protein kinase by glucose-6-phosphate. Plant Physiol. 123, 403-412.

Torres, N.V., Mateo, F., Melendez-Hevia, E., 1988. Shift in rat liver glycolysis control from fed to starved conditions. Flux control coefficients of glucokinase and phosphofructokinase. FEBS Lett. 233, 83-86

Toufighi, K., Brady, S.M., Austin, R., Ly, E., Provart, N.J., 2005. The Botany Array Resource: e-Northerns, Expression Angling, and promoter analyses. Plant J. 43, 153-163.

Trethewey, R.N., Riesmeier, J.W., Willmitzer, L., Stitt, M., Geigenberger, P., 1999. Tuber-specific expression of a yeast invertase and a bacterial glucokinase in potato leads to an activation of sucrose phosphate synthase and the creation of a sucrose futile cycle. Planta 208, 227-238.

Tsujimoto,Y., Izawa,S., Inoue,Y., 2000. Cooperative regulation of DOG2, encoding 2-deoxyglucose-6-phosphate phosphatase, by Snf1 kinase and the high-osmolarity

glycerol-mitogen-activated protein kinase cascade in stress responses of Saccharomyces cerevisiae. J. Bacteriol. 182, 5121-5126.

Turgeon, R., 1996. Phloem loading and plasmodesmata. Trends Plant Sci. 1, 418-423.

Turner, J.F., Chensee, Q.J., Harrison, D.D., 1977. Glucokinase of pea seeds. Biochim. Biophys. Acta 480, 367-375.

Turner, J.F., Copeland, L., 1981. Hexokinase II of pea seeds. Plant Physiol. 68, 1123-1127.

Urbanczyk-Wochniak, E., Leisse, A., Roessner-Tunali, U., Lytovchenko, A., Reismeier, J., Willmitzer, L., Fernie, A.R., 2003. Expression of a bacterial xylose isomerase in potato tubers results in an altered hexose composition and a consequent induction of metabolism. Plant Cell Physiol. 44, 1359-1367.

Veramendi, J., Fernie, A.R., Leisse, A., Willmitzer, L., Trethewey, R.N., 2002. Potato hexokinase 2 complements transgenic *Arabidopsis* plants deficient in hexokinase 1 but does not play a key role in tuber carbohydrate metabolism. Plant Mol. Biol. 49, 491-501.

Veramendi, J., Roessner, U., Renz, A., Willmitzer, L., Trethewey, R.N., 1999. Antisense repression of hexokinase 1 leads to an overaccumulation of starch in leaves of transgenic potato plants but not to significant changes in tuber carbohydrate metabolism. Plant Physiol. 121, 123-134.

Vijn, I., Smeekens, S., 1999. Fructan: more than a reserve carbohydrate? Plant Physiol. 120, 351-360.

Von Euler, H., Adler, E., 1935. Über die Komponenten der Dehydrasesysteme. 6. Dehyrierung von Hexosen unter Mitwirkung von Adenosintriphosphorsäure. Z. Physiol. Chem. 235, 122-153

Wang, H., Iynedjian, P.B., 1997. Modulation of glucose responsiveness of insulinoma beta-cells by graded overexpression of glucokinase. Proc. Natl. Acad. Sci. USA 94, 4372-4377.

Weber, H., Borisjuk, L., Wobus, U., 1997. Sugar import and metabolism during seed development. Trends Plant Sci. 2, 169-174.

Weber, H., Borisjuk, L., Wobus, U., (2005) Molecular physiology of legume seed development. Ann. Rev. Plant Biol. 56, 253-279

Weber, A., Servaites, J.C., Geiger, D.R., Kofler, H., Hille, D., Groner, F., Hebbeker, U., Flügge, U.I., 2000. Identification, purification, and molecular cloning of a putative plastidic glucose translocator. Plant Cell 12, 787-801.

Weise, S.E., Weber, A.P., Sharkey, T.D., 2004. Maltose is the major form of carbon exported from the chloroplast at night. Planta 218, 474-482.

Weschke, W., Panitz, R., Gubatz, S., Wang, Q., Radchuk, R., Weber, H., Wobus, U., 2003. The role of invertases and hexose transporters in controlling sugar ratios in maternal and filial tissues of barley caryopses during early development. Plant J. 33, 395-411.

Wiese, A., Groner, F., Sonnewald, U., Deppner, H., Lerchl, J., Hebbeker, U., Flügge, U., Weber, A., 1999. Spinach hexokinase I is located in the outer envelope membrane of plastids. FEBS Lett. 461, 13-18.

Wilson, J.E., 2003. Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. J. Exp. Biol. 206, 2049-2057.

Winter, H., Robinson, D.G., Heldt, H.W., 1994. Subcellular volumes and metabolite concentrations in spinach leaves. Planta 193, 530-535.

Xia, J.H., Saglio, P., Roberts, J.K.M., 1995. Nucleotide levels do not critically determine survival of maize root tips acclimated to a low oxygen environment. Plant Physiol. 108, 589-595.

Xiao, W., Sheen, J., Jang,J. C., 2000. The role of hexokinase in plant sugar signal transduction and growth and development. Plant Mol. Biol. 44, 451-461.

Yamada, K., Lim, J., Dale, J.M., Chen, H., Shinn, P., Palm, C.J., Southwick, A.M., Wu, H.C., Kim, C., Nguyen, M., Pham, P., Cheuk, R., Karlin-Newmann, G., Liu, S.X., Lam, B., Sakano, H., Wu, T., Yu, G., Miranda, M., Quach, H.L., Tripp, M., Chang, C.H., Lee, J.M., Toriumi, M., Chan, M.M., Tang, C.C., Onodera, C.S., Deng, J.M., Akiyama, K., Ansari, Y., Arakawa, T., Banh, J., Banno, F., Bowser, L., Brooks, S., Carninci, P., Chao, Q., Choy, N., Enju, A., Goldsmith, A.D., Gurjal, M., Hansen, N.F., Hayashizaki, Y., Johnson-Hopson, C., Hsuan, V.W., Iida, K., Karnes, M., Khan, S., Koesema, E., Ishida, J., Jiang, P.X., Jones, T., Kawai, J., Kamiya, A., Meyers, C., Nakajima, M., Narusaka, M., Seki, M., Sakurai, T., Satou, M., Tamse, R., Vaysberg, M., Wallender, E.K., Wong, C., Yamamura, Y., Yuan, S., Shinozaki,

K., Davis,R. W., Theologis, A., Ecker, J.R., 2003. Empirical analysis of transcriptional activity in the *Arabidopsis* genome. Science 302, 842-846.

Yamamoto, Y.T., Prata, R.T.N., Williamson, J.D., Weddington, M., Pharr, D.M., 2000. Formation of a hexokinase complex is associated with changes in energy utilization in celery organs and cells. Physiol. Plant. 110, 28-37.

Yanagisawa, S., Yoo, S.D., Sheen, J., 2003. Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature 425, 521-525.

Yoneyama, T., Shiozawa, M., Nakamura, M., Suzuki, T., Sagane, Y., Katoh, Y., Watanabe, T., Ohyama, T., 2004. Characterization of a novel acid phosphatase from embryonic axes of kidney bean exhibiting vanadate-dependent chloroperoxidase activity. J. Biol. Chem. 279, 37477-37484.

Zeeman, S.C., Smith, S.M., Smith, A.M., 2004. The breakdown of starch in leaves. New Phytol. 163, 247-261.

Zrenner, R., Salanoubat, M., Willmitzer, L., Sonnewald, U., 1995. Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). Plant J. 7, 97-107.

Chapitre 3.

Clonage, Expression, Purification et Propriétés d'une Hexokinase Putative de la Membrane Plasmique de *Solanum chacoense*

Claeyssen, Wally, Matton, Morse et Rivoal, 2006. Protein Expr. Purif. 47, 329-339.

Contribution des coauteurs :

Je suis l'auteur principal du texte de cet article. Le clone *ScHK2* utilisé dans ce projet nous a été gracieusement fourni par le Dr. D.P. Matton. Le séquençage de l'ADNc *ScHK2* et la production d'anticorps polyclonal anti-hexokinase (anti-ΔScHK2) ont été effectués par O. Wally. Les Drs. D. Morse et J. Rivoal ont monté la Figure 3.2 et le Tableau 3.1, et contribué substantiellement au texte s'y rattachant. Les autres expériences, données non montrées, figures et tableaux ont été réalisés par moi-même.

3.1 Title page

Cloning, expression, purification and properties of a putative plasma membrane hexokinase from *Solanum chacoense**

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*This work was supported by a Discovery Grant from the National Science and Engineering Research Council of Canada to J. R.

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Submitted to Protein Expression and Purification 13 September 2005.
Accepted in revised form 4 November 2005.
Published online 5 December 2005.
Claeyssen, Wally, Matton, Morse and Rivoal, 2006. Protein Expr. Purif. 47, 329-339.

3.2 Preliminary material

3.2.1 Keywords

Hexokinase; Kinetic analysis; *Solanum chacoense*; Affinity chromatography; *Escherichia coli*; Hexose metabolism; Hexose-phosphates.

3.2.2 Abbreviations

F1P, fructose-1-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; FK, fructokinase; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; GK, glucokinase; HK, hexokinase; IgG, immunoglobulin G; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria–Bertani; Ni–NTA, Ni^{2+}–nitrilotriacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; T6P, trehalose-6-phosphate; U, unit.

3.3 Abstract

A full-length hexokinase cDNA was cloned from *Solanum chacoense*, a wild relative of the cultivated potato. Analysis of the predicted primary sequence suggested that the protein product, ScHK2, may be targeted to the secretory pathway and inserted in the plant plasma membrane, facing the cytosol. ScHK2 was expressed as a hexahistidine-tagged protein in *Escherichia coli*. Expression conditions for this construct were optimized using a specific anti-hexokinase polyclonal anti-serum raised against a truncated version of ScHK2. The full-length recombinant protein was purified to electrophoretic homogeneity using immobilized metal ion affinity chromatography followed by anion exchange chromatography on Fractogel EMD DEAE-650 (S). The purified enzyme had a specific activity of 5.3 μmol/min/mg protein. Its apparent K_ms for glucose (23 μM), mannose (30 μM), fructose (5.2 mM) and ATP (61 μM) were in good agreement with values found in the literature for other plant hexokinases. Hexahistidine-tagged ScHK2 was highly sensitive to pH variations between 7.7 and 8.7. It was inhibited by ADP and insensitive to glucose-6-

phosphate. These findings constitute the first kinetic characterization of a homogeneous plant hexokinase preparation. The relevance of ScHK2 kinetic properties is discussed in relation to the regulation of hexose metabolism in plants.

3.4 Introduction

Hexokinase (HK, EC 2.7.1.1) is distributed in all kingdoms and catalyzes the irreversible, ATP-dependent phosphorylation of several hexoses including glucose, fructose, mannose and galactose. HK is thus distinct from glucokinase (GK, EC 2.7.1.2) and fructokinase (FK, EC 2.7.1.4) that are highly specific to glucose and fructose, respectively (Cárdenas *et al.*, 1998). In plants, glycolysis begins with sucrose degradation either into glucose and fructose by invertase (EC 3.2.1.26), or into UDP-glucose and fructose by sucrose synthase (EC 2.4.1.13) (Plaxton, 1996). Therefore, HK, GK and FK reactions may constitute a potentially important site for regulation of carbon metabolism as they commit hexoses to cell metabolism (Renz *et al.*, 1993; Renz and Stitt, 1993; Martinez-Barajas and Randall, 1998; Pego and Smeekens, 2000).

Plants typically contain an array of several HK isoforms that differ in their chromatographic and kinetic properties (Renz *et al.*, 1993; Renz and Stitt, 1993). Estimated molecular masses range from 38 to 68 kDa (Renz *et al.*, 1993; Higgins and Easterby, 1974; Miernyk and Dennis, 1983; Yamamoto *et al.*, 2000). Among species studied, the K_m for glucose is low and may vary between 15 and 150 µM. In contrast, the K_m for fructose is always in the millimolar range (Higgins and Easterby, 1974; Renz and Stitt, 1993; Dai *et al.*, 1995; Galina *et al.*, 1995; Dai *et al.*, 1999; Veramendi *et al.*, 1999; Menu *et al.*, 2001; Giese *et al.*, 2005). The affinity for ATP (K_m between 50 and 560 µM) (Renz and Stitt, 1993; Galina *et al.*, 1995; Veramendi *et al.*, 1999; da-Silva *et al.*, 2001; Giese *et al.*, 2005) is significantly higher than those of other nucleoside triphosphates (Renz and Stitt, 1993) or comparable to that of UTP (Schnarrenberger, 1990), depending on the isoform considered. In maize roots and potato tubers, only some HK isoforms are inhibited by physiological concentrations of glucose-6-phosphate (G6P) (K_i of 0.03 to 4 mM) or ADP (K_i of 20 to 110 µM) (Renz and Stitt, 1993; Galina *et al.*, 1995; Galina *et al.*, 1999). In addition, regulation

by G6P may be pH-dependent (Renz and Stitt, 1993). HK isoforms may also vary in their subcellular localizations. Active HK isozymes have been reported in the cytosol (Galina *et al.*, 1995), in the Golgi complex (da-Silva *et al.*, 2001), in the nucleus (Sheen *et al.*, 1999), in the chloroplast stroma (Olsson *et al.*, 2003), on the cytosolic side of the chloroplastic outer membrane (Wiese *et al.*, 1999) and of the mitochondrial outer membrane (Giegé *et al.*, 2003).

In addition to its metabolic role in glycolysis, HK is now broadly accepted as a sugar sensor in eukaryotic cells (Sheen *et al.*, 1999; Rolland *et al.*, 2001; Frömmer *et al.*, 2003; Harrington and Bush, 2003). In plants, sugars act as signaling molecules in the control of growth and development during the entire life cycle (Rolland *et al.*, 2002) and regulate expression of genes involved for example in photosynthesis (Jang and Sheen, 1994; Koch, 1996). Studies using sugar analogs, metabolic intermediates and transgenic plants, provided evidence that HK is involved in sugar sensing (Jang and Sheen, 1994; Jang *et al.*, 1997). It has also been shown that sugar sensing by HK in plants was not dependent on HK catalytic activity *per se*, thereby demonstrating that HK has separate functions in glucose metabolism and sensing (Moore *et al.*, 2003).

The question has often been raised as to how the different HK isoforms of a plant cell contribute to hexose metabolism or sensing (Renz and Stitt, 1993; Martinez-Barajas and Randall, 1998; Fox *et al.*, 1998; Wiese *et al.*, 1999; da-Silva *et al.*, 2001; Harrington and Bush, 2003; Olsson *et al.*, 2003; Giese *et al.*, 2005). Although several plant HKs have been structurally and kinetically characterized (Renz *et al.*, 1993; Renz and Stitt, 1993; Dai *et al.*, 1999; Veramendi *et al.*, 1999; Wiese *et al.*, 1999; Menu *et al.*, 2001; Giese *et al.*, 2005), none of these studies provide evidence that the preparations used for enzyme analyses were electrophoretically pure. As part of our ongoing efforts to better understand the control of hexose metabolism in plants, we describe here the cloning of an HK cDNA from *Solanum chacoense*, a wild relative of the cultivated potato. The corresponding recombinant protein was expressed and purified to electrophoretic homogeneity from *Escherichia coli*. To our knowledge, this is the first kinetic characterization of a pure recombinant plant HK.

3.5 Materials and methods

3.5.1 Materials and chemicals

All buffers, chemicals, reagents and commercial enzymes were of analytical grade and purchased from Sigma Chemical (St-Louis, MO) or Fisher Scientific (Nepean, ON, Canada), unless otherwise stated. PD10 columns used for desalting protein extracts were from G.E. Healthcare (Baie d'Urfé, QC, Canada). Ni^{2+}–nitrilotriacetic acid (Ni–NTA) agarose was from Invitrogen Canada (Burlington, ON, Canada). Platinum *Pfx* used for PCR and the vector pPROEx HT, which contains a (6xHis) tag and a Tobacco Etch Virus (TEV) protease cleavage site, were from Invitrogen Canada. Restriction enzymes were from MBI Fermentas (Burlington, ON, Canada), and Invitrogen Canada. Primers for PCR and sequencing were from Sigma Genosys (The Woodlands, TX). Fractogel EMD DEAE-650 (S) was from VWR (Mississauga, ON, Canada).

3.5.2 cDNA cloning and sequencing

A *S. chacoense* EST collection was generated in the pBK-CMV vector (Stratagene, La Jolla, CA) from weakly-expressed mRNAs in pistil tissues (Lantin *et al.*, 1999). A search for a full-length HK in this EST collection led to the identification of a 1.8 kb cDNA encoding a sequence with significant homology to potato HK2 (UniProt Accession No. Q9SQ76). The cDNA (*ScHK2*) was completely sequenced on both strands using the Big Dye Terminator 2.0 sequencing kit (Perkin-Elmer, Montreal, QC, Canada) and an ABI 377 automated sequencer.

3.5.3 Generation of full-length ScHK2 and truncated ScHK2 (ΔScHK2) constructs for heterologous expression in *E. coli*

To express the full-length construct, ScHK2 cDNA in the pBK-CMV vector was first amplified by PCR with Platinum *Pfx* DNA polymerase using the following primers: forward, 5'-ATGAAGAAGGCGACGGTG-3'; reverse, T7 primer. The resulting fragment

was digested with *Kpn*I and cloned into *Ehe*I/*Kpn*I-digested pPROEX HTb. To express the truncated construct, designated ΔScHK2, the ScHK2 cDNA in pBK-CMV was partially digested with *Eco*RI after complete digestion with *Xho*I, and a 1618-bp restriction fragment encompassing the C-terminal part of ScHK2 was isolated. This fragment was cloned into *Eco*RI/*Xho*I-digested pPROEX HTa. The ligated plasmids were used to transform competent *E. coli* (DH5α strain). Restriction digestions confirmed the presence and correct orientation of the inserts. The full-length construct in the expression plasmid carried the entire coding sequence of ScHK2 in frame with an N-terminal 24-amino acid extension containing the (6xHis) tag from the expression vector. The deduced amino acid sequence of the resulting fusion protein was 520 amino acids long and had a predicted molecular mass of 56,776 Da. For the truncated ScHK2 (ΔScHK2) construct, the expression plasmid carried the coding sequence of the C-terminal part of ScHK2, in frame with a 29-amino acid extension at the N-terminus. This extension contained the (6xHis) tag of the expression vector. The deduced amino acid sequence of the resulting (6xHis)ΔScHK2 protein was 482 amino acids long with a predicted mass of 52,817 Da.

3.5.4 Expression and purification of recombinant proteins

For the full-length recombinant ScHK2, *E. coli* carrying the expression plasmid was grown in Fernbach flasks at 37 °C in Luria–Bertani (LB) broth medium (0.5 L volume) to an A_{600} of 0.5–0.7. At this point, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.6 mM. Unless otherwise mentioned, cultures were left to grow for 1 h at 23 °C. Cells were harvested by centrifugation (15 min at 10,000*g*), and pellets were frozen at –80 °C until used. Purification steps were carried out at 4 °C in native conditions. Bacterial pellets were thawed in 10 mL lysis buffer (50 mM NaH_2PO_4, 10 mM imidazole, 0.3 M NaCl and 14 mM β-mercaptoethanol) adjusted to pH 8.0 with NaOH, and supplemented with 0.5 mM phenylmethylsulfonylfluoride (PMSF), 0.5 mM benzamidine, 0.5 mM ε-amino-*N*-caproic acid (ε-CA), 5 μg/mL leupeptin and 0.05% (w/v) Triton X-100. Bacterial cells were lyzed using a French pressure cell (18,000 psi). After a 15-min centrifugation at 10,000*g*, the supernatant was desalted on PD10 column pre-equilibrated with lysis buffer. The desalted extract was adsorbed in batch on 1 mL

settled volume of Ni–NTA resin pre-equilibrated with lysis buffer during ~75 min. The suspension was then poured into a disposable column (0.5 cm diameter). The column was washed with 16 mL lysis buffer, followed by 12 mL wash buffer (50 mM NaH_2PO_4, 20 mM imidazole, 0.3 M NaCl and 14 mM β-mercaptoethanol). The bound protein was eluted from the column with 2.5 mL elution buffer (50 mM NaH_2PO_4, 250 mM imidazole, 0.3 M NaCl and 14 mM β-mercaptoethanol) and collected in five fractions of 0.5 mL. Fractions containing (6xHis)ScHK2 were pooled and desalted on PD10 column pre-equilibrated with buffer A (20 mM Tris-HCl, pH 8.2, 5 mM $MgCl_2$, 1 mM DTT and 10% [v/v] glycerol), and stored overnight at 4 °C. At this stage, the recombinant enzyme preparation contained several contaminating proteins, so an additional purification step was performed. Pooled desalted fractions were loaded at 0.5 mL/min onto a Fractogel EMD DEAE-650 (S) column (1 x 8 cm) pre-equilibrated in buffer A. The column was connected to a Pharmacia FPLC system and washed with 18 mL buffer A. (6xHis)ScHK2 was eluted with a 72-mL linear gradient of 0–500 mM KCl in buffer A, followed by a step gradient to 1 M KCl in buffer A. One-milliliter fractions were collected and assayed. ScHK2 activity eluted as one peak between 300 and 425 mM KCl. Aliquots of fractions with HK activity were further analyzed by SDS–PAGE and silver staining (Wray *et al.*, 1981) as well as immunodetection (see below). Fractions that displayed HK activity and a single band on silver-stained SDS–PAGE gel were concentrated over a Centricon YM30 filter (Millipore, Nepean, ON, Canada). Because small amounts of protein were recovered, protein amounts in concentrated solution were determined by spectrophotometry at 205 nm (Stoscheck, 1990) on a Cary 100 spectrophotometer (Varian Canada, St. Laurent, QC, Canada). Glycerol was added to the concentrated ScHK2 solution at a final concentration of 50% (v/v) and the solution was stored at –20 °C for up to 3 days without any loss of activity. For the partially purified fractions, protein concentration was determined according to Bradford (1976), using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Mississauga, ON, Canada) and bovine serum albumin as standard.

For (6xHis)ΔScHK2 protein, *E. coli* (DH5α strain) carrying the expression plasmid was grown at 37 °C in 250 mL LB broth medium and induced with IPTG for 5 h at the same temperature. Cells were harvested by centrifugation (10 min at 10,000*g*), and pellets

were frozen at –80 °C until used. Purification steps were carried out at 4 °C. Cell pellets were thawed in 10 mL lysis buffer containing 50 mM Tris-HCl, pH 8.5, 5 mM DTT, 1 mM PMSF, 1 mM benzamidine, 1 mM ε-CA and 0.1% (w/v) Triton X-100 and disrupted using a French pressure cell (18,000 psi). The extract was centrifuged at 10,000*g* for 25 min. The pellet contained insoluble (6xHis)ΔScHK2 and was resuspended in 10 mL buffer B (6 M urea, 0.1 M NaH_2PO_4 and 10 mM Tris-HCl, pH 8.0). After centrifugation at 10,000*g* for 15 min, the clarified sample was adsorbed batchwise with 1 mL Ni–NTA resin equilibrated in buffer B for 1 h. The suspension was then poured in a column (0.5 cm diameter). The column was washed with 4 mL buffer B, followed by 2 mL buffer B adjusted to pH 6.3. The bound protein was eluted from the column with 2 mL buffer B adjusted to pH 5.9, then 2 mL buffer B adjusted to pH 4.5. During elution, 0.5 mL fractions were collected. ΔScHK2 eluted mainly at pH 4.5, as visualized by SDS–PAGE analysis followed by Coomassie blue staining. Fractions containing ΔScHK2 were pooled and adjusted to pH 7.5 with a solution of 1 M Tris-HCl, pH 8.5, and stored frozen until used.

3.5.5 Production and affinity purification of anti-ΔScHK2 immune serum

Purified (6xHis)ΔScHK2 was dialyzed for 16 h against 50 mM Tris-HCl, pH 8.5, and 5 mM DTT. The 3-mL solution of dialyzed protein was clarified by centrifugation at 13,000*g* for 25 min. The protein was digested with 200 U of recombinant TEV protease (Invitrogen Canada) for 3 h at 30 °C. The digested protein was separated from the tag and the protease by chromatography on Ni–NTA resin according to the manufacturer's indications. Approximately 200 µg de-tagged recombinant ΔScHK2 was produced using this procedure and subjected to further purification using preparative SDS–PAGE and electroelution as described by Dorion *et al.* (2005). Antibodies were raised using a 2-kg New-Zealand White rabbit. After collection of the pre-immune serum, de-tagged recombinant ΔScHK2 (120 µg, emulsified in complete Freund's adjuvant) was injected subcutaneously into the back of the rabbit. Booster injections were done at days 14 and 21 with 40 µg of recombinant ΔScHK2 emulsified in incomplete Freund's adjuvant. Final bleed was performed on day 42 by cardiac puncture. The serum was collected after centrifugation at 1,500*g*, frozen in aliquots in liquid N_2, and kept at –80 °C. Crude anti-

serum was purified by affinity against 15 µg recombinant ΔScHK2 using a method modified from Plaxton (1989). Briefly, the membrane strip corresponding to (6xHis)ΔScHK2 was identified by staining with Ponceau S and cut out from the blot. This strip was incubated for 1 h in 1.5 mL blocking buffer containing Tris-buffered saline Tween 20 (TBST) buffer (0.5 M Tris-HCl, pH 7.6, 1.5 M NaCl, 0.5% [v/v] Tween-20 and 0.2% [w/v] NaN_3) supplemented with 3% (w/v) powdered milk. The blot was then washed 3 times for 1 min with 1.5 mL TBST buffer and incubated for 1 h with 1.2 mL crude anti-serum. After removal of anti-serum, the strip was washed 4 times for 1 min with 1.5 mL TBST buffer, then incubated for 2 min with 1.5 mL of elution buffer (20 mM glycine-HCl, pH 3.0, 0.5 M NaCl, 0.25% [v/v] Tween-20 and 0.04% [w/v] NaN_3). Eluted IgGs were collected, neutralized with a solution of 1 M Tris-HCl, pH 8.5, and adjusted to 0.5% (w/v) BSA. Purified IgGs were frozen in liquid N_2 and stored at –80 °C until used.

3.5.6 SDS–PAGE and immunoblot analysis of ScHK2

SDS–PAGE analyses on 12% acrylamide gels and electrotransfer to polyvinylidene difluoride (PVDF) membranes were performed as described previously (Laemmli, 1970; Rivoal *et al.*, 2001). For immunodetection, blots were incubated with affinity-purified anti-ΔScHK2 IgGs (1/15 dilution). Polypeptides were detected using an anti-rabbit alkaline phosphatase-tagged secondary antibody (1/10,000 dilution) (Promega, Nepean, ON, Canada). The phosphatase reaction was visualized with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT), and allowed to develop for 4 to 30 min at 30 °C. Immunoblots incubated with the pre-immune serum gave negative results (data not shown).

3.5.7 Hexokinase activity assay

HK activity assays were conducted according to a protocol modified from Martinez-Barajas and Randall (1998). The HK reaction was coupled to the glucose-6-phosphate dehydrogenase (EC 1.1.1.49) reaction, and assayed at 30 °C by monitoring NAD^+ reduction

at 340 nm using a VersaMax (Molecular Devices, Sunnyvale, CA, USA) microplate reader. The 200-µL reaction mixture contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 0.3 mM NAD^+, 1 mM ATP, 1.4 U/mL glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and (i) 5 mM glucose for glucokinase (EC 2.7.1.2) activity, (ii) 6.7 U/mL phosphoglucose isomerase (EC 5.3.1.9) and 5 mM fructose for fructokinase (EC 2.7.1.4) activity, or (iii) 3.5 U/mL phosphoglucose isomerase, 3.5 U/mL phosphomannose isomerase (EC 5.3.1.8), and 5 mM mannose for mannokinase (EC 2.7.1.7) activity. Reaction rates were linear with time and proportional to the amount of enzyme added to the assay within a range spanning one order of magnitude (data not shown). To determine HK activity towards 3-*O*-methylglucose and to study HK inhibition by glucose-6-phosphate, a coupled-enzyme assay with pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.28) was used. The conditions to measure initial rates of ADP production in this assay were adapted from Martinez-Barajas and Randall (1998). The assay mixture (200 µL) contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 0.15 mM NADH, 1 mM ATP, 0.5 mM phospho*enol*pyruvate, 1 mM glucose (or 100 mM 3-*O*-methylglucose), 3.1 U/mL pyruvate kinase and 15.4 U/mL lactate dehydrogenase. Assays were initiated by addition of enzyme preparation and corrected for background activity by omitting substrate from the reaction mixture. One unit (U) of HK activity is defined as the amount of enzyme that catalyzes the production of 1 µmol of G6P or ADP per min at 30 °C.

3.5.8 Characterization of ScHK2 kinetic properties

For all analyses, presented data are means ± SE of determinations carried out on three independent enzyme preparations with quadruplicate assays unless otherwise mentioned. The effect of pH on ScHK2 activity was studied using a three-component buffer to maintain a constant ionic strength throughout the pH range (Ellis and Morrison, 1982). The Tris-HCl buffer used in the standard reaction mixture described above was therefore replaced with 0.05 M acetic acid, 0.05 M 2-(*N*-morpholino)-ethanesulfonic acid (MES) and 0.1 M Tris-HCl adjusted at different pH values with 1 M NaOH or HCl. The assay pH was measured directly in the reaction mixture using a microelectrode immediately after

completion of the spectrophotometric assay. Apparent K_m (K_{mapp}) values were calculated from the Michaelis–Menten equation using a non-linear least-squares regression program (SigmaPlot 8.0, SPSS, Chicago, IL, USA). *k*cat values were calculated from the V_{max} of Michaelis–Menten plots and using a subunit molecular mass of 56,776 Da.

3.6 Results and discussion

3.6.1 Characteristics of the protein sequence encoded by *ScHK2* and implications for the production of a recombinant ScHK2 carrying a (6xHis) epitope tag at the N-terminus

A full-length cDNA encoding ScHK2 was isolated from *S. chacoense* (GenBank Accession No. DQ177440). The cDNA was 1793 nt long and contained a 496 amino acid ORF. Deduced amino acid sequence from the ORF predicted a 53,751 Da polypeptide with an isoelectric point of 6.07. Analysis of the polypeptide sequence (Fig. 3.1) showed a high degree of amino acid sequence similarity (>97%) with *Nicotiana sylvestris* HK3, *S. tuberosum* HK2 and *Solanum esculentum* HK2, which are all members of the *Solanaceae* family.

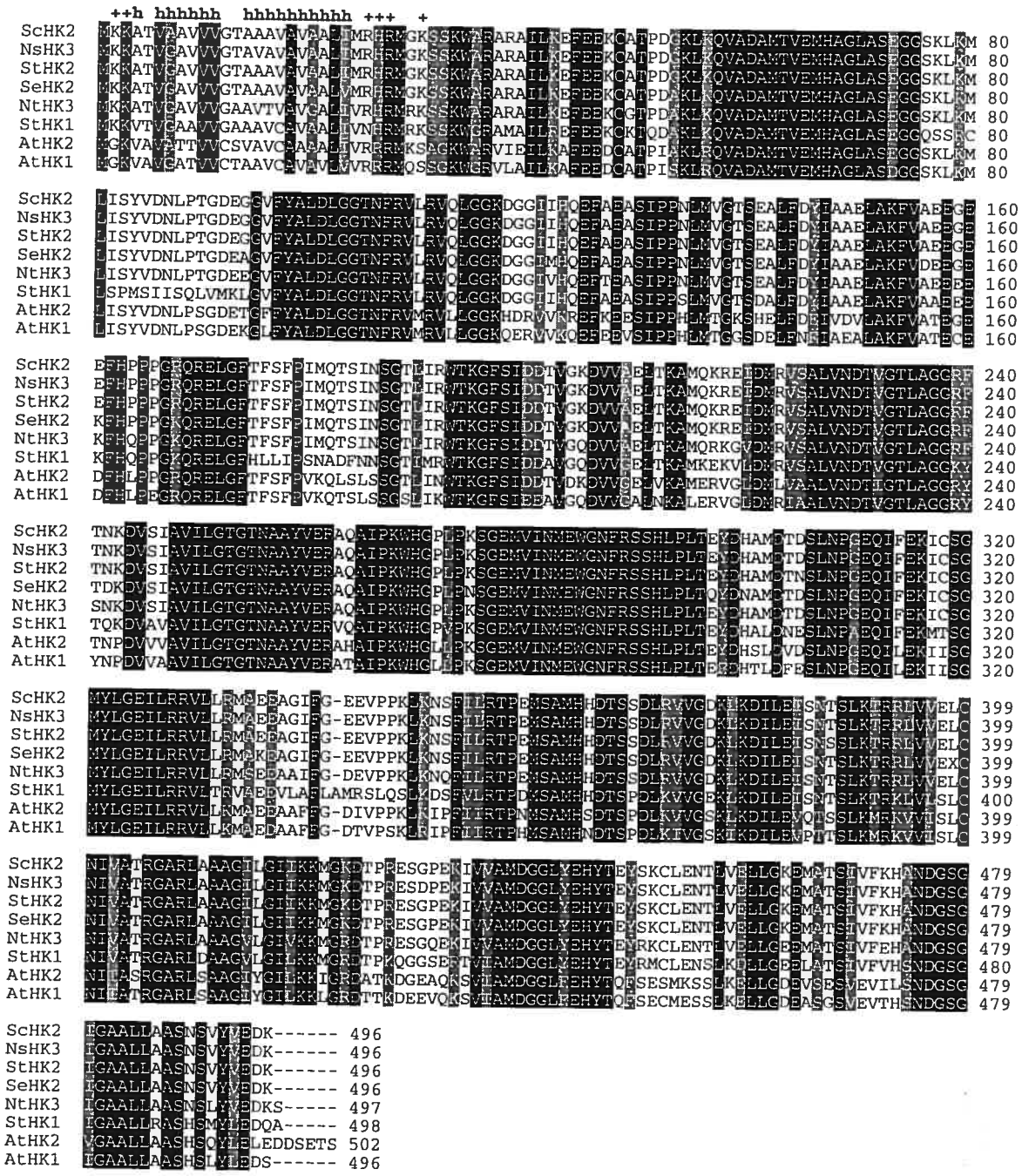
++h hhhhhh hhhhhhhhhh +++ +
ScHK2
NsHK3
StHK2
SeHK2
NtHK3
StHK1
AtHK2
AtHK1

Figure 3.1: Multiple amino acid sequence alignment of eight plant HKs.

The derived amino acid sequence of the *S. chacoense* HK2 cDNA was compared to those of other plant HKs using CLUSTAL W (1.74). Identical and similar amino acid residues are darkened and shaded, respectively. The distribution of basic (+) and hydrophobic (h) amino acid residues in the N-terminal region of ScHK2 is indicated. Accession numbers of sequences used here are as follows: *S. chacoense* ScHK2 (GenBank DQ177440), *N. sylvestris* NsHK3 (UniProt Q6BDB7), *S. tuberosum* StHK2 (UniProt Q9SQ76), *S. esculentum* SeHK2 (UniProt Q9FR27), *N. tabacum* NtHK3 (UniProt Q6Q8A4), *S. tuberosum* StHK1 (UniProt O64390), *A. thaliana* AtHK2 (UniProt P93834) and *A. thaliana* AtHK1 (UniProt Q42525). Note: NsHK3, SeHK2, NtHK3, AtHK2 and AtHK1 are named NsHxk3, LeHxk2, NtHxk3, AtHXK2 and AtHXK1, respectively, in the other chapters of this thesis. ScHK2, StHK2 and StHK1 have the same names in all chapters.

The existence of stromal and membrane-bound HKs in plants (Olsson *et al.*, 2003; Wiese *et al.*, 1999) as well as the fact that there was a stretch of hydrophobic amino acids at the N-terminus of the protein (Fig. 3.1) prompted us to investigate the predicted subcellular localization of ScHK2 (Table 3.1). The sequence was analyzed with TargetP 1.1 (Emanuelsson *et al.*, 2000) available at http://www.cbs.dtu.dk/services/TargetP/, Protein Prowler 1.1 (Bodén and Hawkins, 2005) available at http://pprowler.imb.uq.edu.au and PSORT (Nakai and Kanehisa, 1991) available at http://psort.ims.u-tokyo.ac.jp/ (Table 3.1). Targeting of ScHK2 to the secretory pathway and endoplasmic reticulum membrane obtained the highest scores in these analyses. Targeting to other cell compartments including the mitochondrion or the chloroplast, appeared much less plausible (Table 3.1). The SignalP 3.0 program (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1997) available at http://www.cbs.dtu.dk/services/SignalP/ predicted a signal peptide at the N-terminus, and a possible cleavage site between amino acid residues 20 and 21. Signal peptides are known to control the entry of eukaryotic and prokaryotic proteins in the secretory pathway. Typical secretory pathway signal peptides contain 1 or more basic residues at the N-terminus, followed by a hydrophobic region of 7 or more amino acids and a neutral polar region that may be recognized by leader peptidases (Nielsen *et al.*, 1997; Baneyx, 1999). These features are consistent with the two basic Lys residues at positions 2 and 3, the hydrophobic region between positions 6 and 17 and the putative signal peptidase site AVA between residues 18 and 20 in the ScHK2 sequence (Fig. 3.1). However, this topology predicts that ScHK2 is secreted in the plant apoplastic fluid. The secretion of ScHK2 outside of the cell seems improbable since (i) secretion of HK has never been described and (ii) there is no known source of ATP in the plant apoplast. It thus seems more likely that the signal peptide continues on to residue 23. In this case, the hydrophobic region would be followed by a more basic region (MRHRMGK) than that found at the N-terminus (Fig. 3.1). The remainder of the protein is predominantly hydrophilic (Fig 3.2A) and does not present any evidence of another transmembrane domain. According to the 'positive-inside rule' (Higy *et al.*, 2004), these particular sequence characteristics are consistent with the prediction that in plant cells, ScHK2 would be anchored by its N-terminal domain in the plasma membrane, with the bulk of the protein facing the cytoplasm (Fig. 3.2B).

The construction of the full-length recombinant protein added the sequence NH_2-MSYYHHHHHHDYDIPTTETLYFQG- to the N-terminus of ScHK2. The stretch of His residues places a predominantly positively charged region upstream of the signal peptide and might be expected to have a topology exactly opposite to that of the native protein (Higy *et al.*, 2004). Efficient translocation of heterologous proteins is possible in *E. coli* (Baneyx, 1999) and we would predict that (6xHis)ScHK2 could behave as a secretory protein.

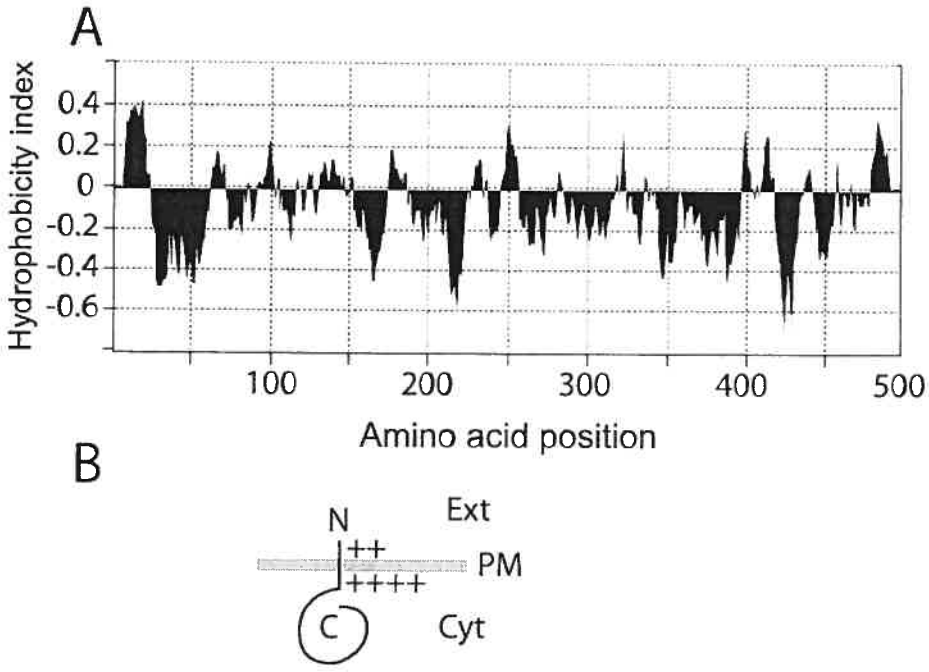

Figure 3.2: Hydrophobicity plot and predicted topology of ScHK2.

(A) hydrophobicity index was calculated with Mac vector 7.0 and plotted as a function of amino acid position. (B) Proposed topology of ScHK2 in the plasma membrane. The scheme represents ScHK2 anchored in the plasma membrane (PM). ScHK2 N- and C-termini are respectively localized in the extracellular (Ext) and cytoplasmic (Cyt) compartments. The proposed localization of N-terminal domain basic residues (+) on each side of the membrane is also depicted.

Table 3.1: Prediction of the subcellular targeting for the ScHK2 protein sequence in plants.

Program	Subcellular targeting or localization	Score
TargetP 1.1	cTP	0.15
	mTP	0.13
	SP	**0.68**
	other	0.02
Protein Prowler 1.1	cTP	0.00
	mTP	0.04
	SP	**0.91**
	other	0.04
PSORT	**ERM**	**0.82**
	ERL	0.10
	PM	0.19
	EC	0.10

The deduced primary sequence was analyzed using TargetP 1.1, PSORT and Protein Prowler 1.1. The scores indicate the likelihood of targeting to a particular compartment by the different programs. The predicted subcellular localization for each program appears in boldface. cTP, chloroplastic targeting; mTP, mitochondrial targeting; SP, secretory pathway; ERM, endoplasmic reticulum membrane; ERL, endoplasmic reticulum lumen; PM, plasma membrane; EC, extracellular.

3.6.2 Expression and purification of recombinant ScHK2

Our first attempts to express (6xHis)ScHK2 in *E. coli* cells were met with limited success (Fig 3.3A), in agreement with our prediction that (6xHis)ScHK2 secretion could interfere with accumulation of the protein. We thus constructed a truncated form of the protein lacking the first 47 amino acids of ScHK2 (ΔScHK2). This deletion removed the entire predicted signal peptide. The ΔScHK2 construct could be expressed in bacteria to levels detectable by SDS–PAGE analysis (Fig. 3.3B), however, the protein was insoluble and inactive. We nevertheless took advantage of this high level of expression to raise an anti-serum against ΔScHK2. This immune serum was used to optimize the conditions for production of the full-length (6xHis)ScHK2.

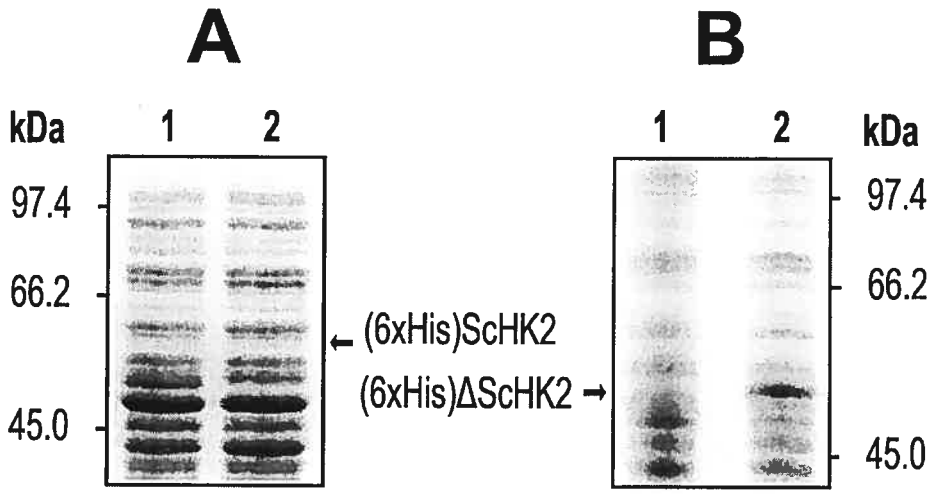

Figure 3.3: SDS–PAGE analysis of (6xHis)ScHK2 (A) and (6xHis)ΔScHK2 (B) expression in *E. coli*.

Lane 1: non-induced, total crude extract. Lane 2: total crude extract after 4 h induction at 37°C with IPTG. The running positions of molecular mass standards are indicated on the left, those of ScHK2 and ΔScHK2 are indicated by arrows. Fifty microliters of culture were loaded on each lane.

Using the anti-ΔScHK2 immune serum, the steady-state levels of (6xHis)ScHK2 were monitored in *E. coli* cells and in the culture medium over an induction period of 15 to 240 min at 23 and 37 °C (Figs. 3.4A and B). The band present in non-induced bacteria (– IPTG lane), may reflect leakiness in the control of the *trc* promoter over ScHK2 expression. Nonetheless, induction of (6xHis)ScHK2 expression was efficient as evidenced by more intense bands in +IPTG lanes than in the –IPTG lane (Fig. 3.4A). ScHK2 steady-state levels in cells induced for 15 to 60 min were higher at 37 than at 23 °C (Fig. 3.4A). However, those levels declined beyond 60 min of induction, with a more dramatic effect at 37 °C. This decline coincided with rising ScHK2 levels in the culture medium, especially at 37 °C (Fig. 3.4B). We therefore investigated HK activity in bacteria and in their culture medium after 240 min of induction (Fig. 3.4C). Levels of HK activity were extremely low in bacteria and in the medium when induction of ScHK2 expression was performed at 37 °C. Contrastingly, HK activity was much higher (5.6 mU/mL of culture) in bacteria induced at 23 °C. HK activity was slightly higher in the culture medium than in cells at 37 °C, in accordance with the immunoblot data. However, HK activity levels in the culture medium remained much lower than the levels found in cells at 23 °C. Taken together, these data suggest that ScHK2 was secreted from *E. coli* cells during induction, and that this effect was more pronounced at 37 than at 23 °C. For reasons that we did not investigate further, cell cultures induced at 37 °C displayed less HK activity than those induced at 23 °C. Lastly, secretion of ScHK2 into the culture medium seemed to result in a loss of activity. Based on these results, we decided to purify the ScHK2 protein from *E. coli* cell cultures induced for 60 min at 23 °C. Indeed, these conditions produced active recombinant ScHK2 and produced the strongest immunoblot signal during the induction time course.

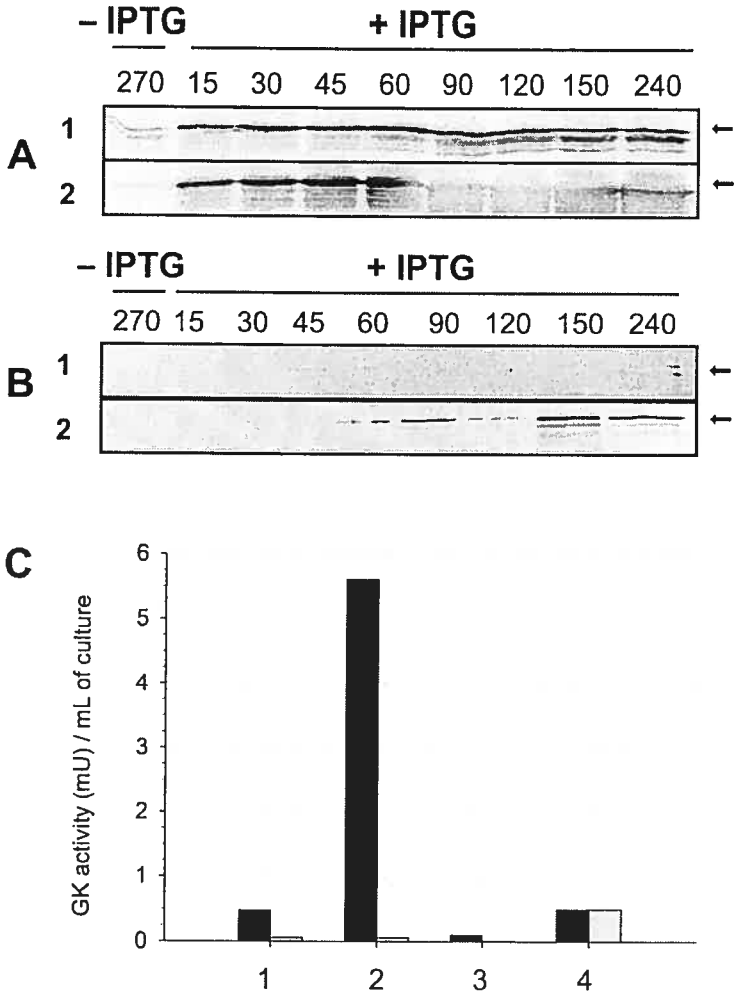

Figure 3.4: Immunoblot analysis (A and B), and HK activity assay (C) on *E. coli* cell cultures expressing (6xHis)ScHK2.

(A) Immunoblot analysis of ScHK2 expressed in *E. coli* cell pellets after induction of the cultures with or without IPTG for 15–270 min at 23 °C (blot 1) or at 37 °C (blot 2). Control (non-induced) cells are analyzed on the (–IPTG) lane. (B) Immunoblot analysis of ScHK2 in culture medium of *E. coli* cell cultures used in (A). The running position of ScHK2 is indicated by an arrow on the various blots. The lanes contain equivalents of 50 µL of culture in (A) and of 18 µL of culture in (B). (C) HK activity was assayed in bacterial cell extracts and in the culture medium concentrated over a Centricon YM3 membrane. Data are representative of at least two separate experiments for cultures incubated with or without IPTG for 240 min. Black bars, induction at 23 °C. Grey bars, induction at 37 °C. 1, –IPTG cells; 2, +IPTG cells; 3, medium of –IPTG cells; 4, medium of +IPTG cells.

Results of a typical purification procedure are presented in Table 3.2. The (6xHis)ScHK2 protein was purified over 260 fold to a specific activity of 5.3 U/mg protein. To our knowledge, this corresponds to the highest specific activity ever reported for a plant HK (Turner and Copeland, 1981; Copeland and Morell, 1985; Doehlert, 1989; Renz and Stitt, 1993; Galina *et al.*, 1995; Veramendi *et al.*, 1999; Wiese *et al.*, 1999; da-Silva *et al.*, 2001; Menu *et al.*, 2001). (6xHis)ScHK2 was purified to electrophoretic homogeneity as shown by silver staining of SDS gel (Fig. 3.5, lane 1). An immunoblot analysis carried out on the purified preparation (Fig. 3.5, lane 2) showed that purified (6xHis)ScHK2 was free of degradation products. This purified protein was used to conduct kinetic studies.

Table 3.2: Purification of recombinant ScHK2 from *E. coli* cells.

Step	Total activity (U) [a]	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
desalted extract	2.3	107.2	0.02	100	1
Ni–NTA	0.5	0.7	0.71	22	35.5
DEAE Fractogel	0.10	0.02	5.29	4	265

[a] Activity unit expressed in µmol/min with glucose as substrate.

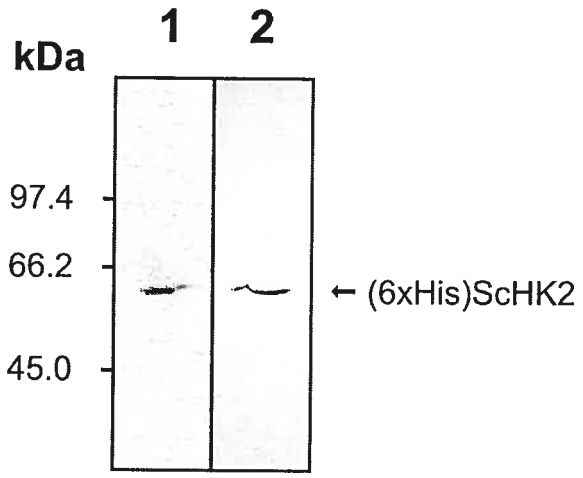

Figure 3.5: Analysis of purified (6xHis)ScHK2 by SDS–PAGE (1) and immunoblot (2).

The gel was silver-stained as described in Material and methods. Immunoblot was carried out using affinity-purified IgGs. The running positions of molecular mass standards are indicated on the left.

3.6.3 Effects of pH on ScHK2 activity

ScHK2 displayed an optimum pH at the fairly high pH value of 8.7 (Fig. 3.6). Moreover, ScHK2 activity decreased sharply (3-fold) within a narrow range from pH 8.7 down to pH 7.7. Such a sharp pH curve resembled that of HK1 in potato tuber (Renz and Stitt, 1993; Veramendi *et al.*, 1999) but contrasted with the broad pH response observed for most plant HKs (Turner and Copeland, 1981; Copeland and Morell, 1985; Doehlert, 1989). Therefore, ScHK2 activity may be quite responsive to slight changes in physiological pH.

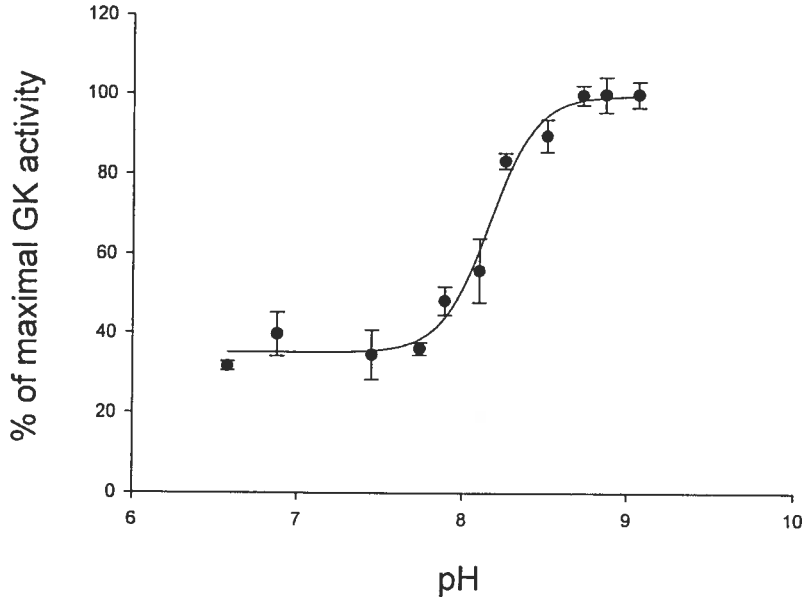

Figure 3.6: Effects of pH variation on ScHK2 activity.

Values are representative of data from two separate purifications (100% = 3.1 mU using glucose as substrate).

3.6.4 Kinetic behavior of ScHK2

We determined the kinetic constants of pure (6xHis)ScHK2 for various substrates (Table 3.3). Recombinant ScHK2 protein exhibited comparable affinity for glucose (K_{mapp} = 23 µM) and mannose (K_{mapp} = 30 µM). The enzyme could phosphorylate both aldoses with a relatively high catalytic efficiency (k_{cat}/K_{mapp} > 120 mM^{-1} s^{-1}). In contrast, ScHK2 had lower affinity for fructose (K_{mapp} = 5.2 mM) and a catalytic efficiency that was more than 100-fold lower than with glucose and mannose. Therefore, (6xHis)ScHK2 displayed typical kinetic characteristics of a plant HK (Copeland and Morell, 1985; Renz and Stitt, 1993; Wiese *et al.*, 1999). In heterotrophic organs such as potato tuber, cytosolic glucose concentrations can reach 30 mM while concentrations of mannose and fructose are respectively more than three orders of magnitude lower and not detected (Farré *et al.*, 2001). Consequently, phosphorylation of glucose in tubers by ScHK2 is most likely not limited by *in vivo* glucose concentrations. Among nucleoside triphosphates, ATP was the preferred substrate (K_{mapp} = 61 µM) with a catalytic efficiency that was at least 10 times higher than with other nucleoside triphosphates (Table 3.3). Similar results were obtained with HKs purified from potato tuber (Renz and Stitt, 1993) and from developing maize kernels (Doehlert, 1989). Farré *et al.* (2001) reported ATP cytosolic concentrations in excess of 200 µM ATP in potato tuber cells, and developing wheat seeds contain 250 nmol/g FW (van Dongen *et al.*, 2004). ATP is therefore probably not limiting for ScHK2 in plant cells under normal conditions. The K_{mapp} (UTP) was in the millimolar range. Despite the fact that UTP is generally fairly abundant (Farré *et al.*, 2001; van Dongen *et al.*, 2004), the estimate of its cytosolic concentration is still much lower than the K_{mapp}. It is therefore doubtful that UTP plays a significant role in hexose phosphorylation by ScHK2.

Table 3.3: Kinetic constants of pure ScHK2 [a].

Substrate	K_{mapp} (mM)	k_{cat} (s^{-1})	k_{cat}/K_{mapp} ($mM^{-1}\ s^{-1}$)
D-glucose	0.023 ± 0.003	5.01 ± 0.75	218
D-fructose	5.2 ± 0.4	3.78 ± 1.13	0.7
D-mannose	0.030 ± 0.004	3.69 ± 0.47	123
ATP	0.061 ± 0.01	3.50 ± 0.37	57
TTP	0.231	1.42	6.1
GTP	0.231	0.85	3.7
UTP	2.2	3.02	1.4
CTP	2.0	1.61	0.8

[a] For hexoses and ATP, kinetic parameters are given as means ± SE of values from three independent enzyme preparations. Values for TTP, GTP, UTP, and CTP, are representative of two independent enzyme preparations.

We next tested the effect of various compounds on (6xHis)ScHK2 activity. The choice of effectors was made either because of their physiological relevance, or because of their use in studies on the role of HK in sugar sensing (Table 3.4). Amongst them, fructose, sucrose, fructose-1-phosphate (F1P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6BP), and glucose-1-phosphate (G1P) were without any important effect on (6xHis)ScHK2 activity. On the contrary, the recombinant protein was strongly inhibited by mannose, most plausibly due to the high affinities of (6xHis)ScHK2 for both glucose and mannose (Table 3.3). 3-*O*-methylglucose was not an inhibitor. However, it could serve as substrate for (6xHis)ScHK2 (data not shown), thus corroborating previous observations by Cortès *et al.* (2003). Trehalose-6-phosphate (T6P) has been proposed to regulate plant glycolysis through inhibition of HK activity (Paul *et al.*, 2001; Schluepmann *et al.*, 2003), by analogy to the yeast model (Thevelein and Hohmann, 1995). However, we did not observe any inhibition by T6P (Table 3.4), similarly to previous observations with other plant HKs (Wiese *et al.*, 1999; Eastmond *et al.*, 2002; Gonzali *et al.*, 2002). Glucosamine, a specific inhibitor of HK (Salas *et al.*, 1965), has been used in studies investigating the implication of HK in sugar sensing (Jang and Sheen, 1994; Guglielminetti *et al.*, 2000; da-Silva *et al.*, 2001). (6xHis)ScHK2 was more sensitive to glucosamine than other plant HKs characterized so far (Wiese *et al.*, 1999; Guglielminetti *et al.*, 2000; Menu *et al.*, 2001), with 50% inhibition at 5 mM. Interestingly, da-Silva *et al.* (2001) noted that a membrane-bound HK was more sensitive to glucosamine than a soluble isoform in maize roots. In contrast to mammalian HKs, yeast and most plant enzymes are not subject to feedback regulation by G6P (Schluepmann *et al.*, 2003). Nevertheless, some pH-dependent inhibition by G6P has been observed for potato tuber HK1 (Renz and Stitt, 1993) as well as for tomato fruit GK (Martinez-Barajas and Randall, 1998). In our study, we used up to 10 mM G6P since cytosolic levels of 0.4–12 mM have been measured or assumed for different plant tissues (Gerhardt *et al.*, 1987; Renz and Stitt, 1993; Farré *et al.*, 2001). G6P did not inhibit ScHK2 at pH 8.0 (Table 3.4), in agreement with data on some other plant HKs (Copeland and Morell, 1985; Doehlert, 1989; Renz and Stitt, 1993; Galina *et al.*, 1995). There was even a slight activity increase at high G6P concentrations. Similar results were obtained at pH 7.0 (data not shown). Lastly, we observed a strong inhibitory effect of ADP since 0.05–2.5 mM ADP inhibited ScHK2 activity by 40–90% (Table 3.4). Strong

inhibition by ADP has been reported elsewhere (Galina *et al.*, 1995; Galina *et al.*, 1999; da-Silva *et al.*, 2001; Menu *et al.*, 2001). This finding may suggest a major role for the ATP/ADP ratio in regulating ScHK2 activity in plants (da-Silva *et al.*, 2001).

Table 3.4: Effect of various metabolites on (6xHis)ScHK2 activity [a].

Effector added	Effector concentration (mM)	% Activity mean ± SE
none	– –	100 ± 3
D-fructose	1	92 ± 1.4
	10	94 ± 1.2
D-mannose	1	46 ± 1.4
	10	12 ± 1.5
D-sucrose	1	91 ± 0.4
	10	91 ± 2.2
D-fructose-1-phosphate	0.1	90 ± 0.2
	0.25	86 ± 1.5
	0.5	93 ± 4.9
	1	101 ± 0.2
D-fructose-6-phosphate	1	96 ± 1.2
	5	98 ± 1.9
	10	100 ± 0.5
D-fructose-1,6-bisphosphate	1	88 ± 4.7
	10	101 ± 3.1
D-glucose-1-phosphate	1	95 ± 1.4
	10	104 ± 1.5

D-glucose-6-phosphate	0	101 ± 0.6
	0.05	92 ± 0.1
	0.1	93 ± 3.1
	0.5	97 ± 3.8
	1	97 ± 1.7
	5	116 ± 4.5
	10	128 ± 5.5
D-trehalose-6-phosphate	0.2	93 ± 0.6
	0.5	92 ± 0.6
	1	94 ± 1
D-3-*O*-methylglucose	1	91 ± 0.7
	10	89 ± 1.3
	100	92 ± 1.6
glucosamine	0.5	90 ± 2.4
	1	82 ± 6.0
	5	54 ± 3.8
	20	33 ± 2.4
	50	20 ± 2.0
	100	13 ± 2.5
ADP	0.05	57 ± 1.3
	0.1	41 ± 0.9
	0.5	17 ± 0.9
	1	13 ± 0.8
	2.5	11 ± 0.5

[a] Listed metabolites were analyzed in the standard assay medium with a saturating concentration of glucose. The effect is reported as a percentage of the control (100% = 30 mU). Activities are given as means ± SE of values from three independent enzyme preparations. For analyzing the effect of glucose-6-phosphate, a different coupled enzyme assay using PK and LDH was utilized. The 0 mM control for this assay is included in the table.

3.7 Conclusions

Despite its cardinal importance in plant carbohydrate metabolism and sugar sensing, an HK of plant origin had never been previously purified to homogeneity. We circumvented the initial difficulties in the production of (6xHis)ScHK2 by expression of a truncated form of the protein in *E. coli*. Removal of the first 47 amino acids in the construct resulted in high levels of expression for (6xHis)ΔScHK2 that permitted the generation of an anti-HK immune serum. By using affinity-purified anti-HK IgGs and HK activity assays we were able to address the reasons causing low levels of (6xHis)ScHK2 expression. We found that (6xHis)ScHK2 was produced and secreted into the culture medium at 37 °C, although the protein was inactive at that temperature. When expressed at 23 °C, (6xHis)ScHK2 was active and accumulated in modest but detectable levels in cells during the first hour of induction. These conditions were chosen to produce an electrophoretically homogeneous recombinant plant HK preparation for characterization.

Analysis of the ScHK2 sequence predicted that the protein be membrane-anchored by its N-terminal hydrophobic region. In the absence of any additional targeting cues, the most likely destination of ScHK2 is the plasma membrane. Wiese *et al.* (1999) described the first membrane-localized plant HK. This isoform was inserted into the chloroplast outer envelope, facing the cytosolic side in spinach leaf cells. The authors suggested that the protein could be involved in the energization of glucose exported from plastids into the cytosol. By analogy, we propose that ScHK2 constitutes a major control step in hexose import into sink cells, a role that would reflect its predicted subcellular localization and topology. It is worth noting that the features discovered in the N-terminal region of ScHK2 are also present on other plant HKs (see Fig. 3.1). By phosphorylating fructose and particularly glucose produced from sucrose degradation, ScHK2 may help maintain a concentration gradient of these hexoses across the plasma membrane, thereby facilitating their import into the cytosol (Lalonde *et al.*, 2004). Such import would rely on adequate energy charge as ScHK2 may be highly sensitive to rising cytosolic ADP levels (Table 3.4). This would ensure that feeding of cytosolic glycolysis with hexoses is coupled to ATP generation by mitochondrial oxidative phosphorylation. We further hypothesize that hypoxic conditions may be detrimental to the function of ScHK2. Indeed, under oxygen

deprivation, cytosolic ADP levels increase 2–3 fold to 200 µM (Hooks *et al.*, 1994), while cytosolic pH drops from pH 7.5 to 6.5 (Roberts *et al.*, 1984). In anoxic maize root tips,. Bouny and Saglio (1996) have identified hexose phosphorylation as a major controlling step in hexose catabolism. If ScHK2 is present in hypoxic plant tissues, its inhibition by ADP and acidic pH conditions may very well participate to a decrease in hexose phosphorylation. The expression pattern of ScHK2 in plant tissues is still unknown but the estimation of HK protein steady-state levels will be facilitated by the immune serum generated in this study.

3.8 References

Baneyx, F., 1999. Recombinant protein expression in *Escherichia coli*. Curr. Opin. Biotechnol. 10, 411-421.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783-795.

Bodén, M., Hawkins, J., 2005. Prediction of subcellular localization using sequence-biased recurrent networks. Bioinformatics 21: 2279-2286.

Bouny, J.M., Saglio, P.H., 1996. Glycolytic flux and hexokinase activities in anoxic maize root tips acclimated by hypoxic pretreatment. Plant Physiol. 111, 187-194.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Cárdenas, M.L., Cornish-Bowden, A., Ureta, T., 1998. Evolution and regulatory role of the hexokinases. Biochim. Biophys. Acta 1401, 242-264.

Copeland, L., Morell, M., 1985. Hexose kinases from the plant cytosolic fraction of soybean nodules. Plant Physiol. 79, 114-117.

Cortès, S., Gromova, M., Evrard, A., Roby, C., Heyraud, A., Rolin, D.B., Raymond, P., Brouquisse, R.M., 2003. In plants, 3-*O*-methylglucose is phosphorylated by hexokinase but not perceived as a sugar. Plant Physiol. 131, 824-837.

Dai, N., Schaffer, A., Petreikov, M., Shahak, Y., Giller, Y., Ratner, K., Levine, A., Granot, D., 1999. Overexpression of *Arabidopsis* hexokinase in tomato plants inhibits

growth, reduces photosynthesis, and induces rapid senescence. Plant Cell 11, 1253-1266.

Dai, N., Schaffer, A.A., Petreikov, M., Granot, D., 1995. *Arabidopsis thaliana* hexokinase cDNA isolated by complementation of yeast cells. Plant Physiol. 108, 879-880.

da-Silva, W.S., Rezende, G.L., Galina, A., 2001. Subcellular distribution and kinetic properties of cytosolic and non- cytosolic hexokinases in maize seedling roots: implications for hexose phosphorylation. J. Exp. Bot. 52, 1191-1201.

Doehlert, D.C., 1989. Separation and characterization of four hexose kinases from developing maize kernels. Plant Physiol. 89, 1042-1048.

Dorion, S., Parveen, Jeukens, J., Matton, D.P., Rivoal, J., 2005. Cloning and characterization of a cytosolic isoform of triosephosphate isomerase developmentally regulated in potato leaves. Plant Sci. 168, 183-194.

Eastmond, P.J., van Dijken, A., Spielman, M., Kerr, A., Tissier, A.F., Dickinson, H.G., Jones, J.D., Smeekens, S.C., Graham, I.A., 2002. Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. Plant J. 29, 225-235.

Ellis, K.J., Morrison, J.F., 1982. Buffers of constant ionic strength for studying pH-dependent processes. Methods Enzymol. 87, 405-426.

Emanuelsson, O., Nielsen, H., Brunak, S., von Heijne, G., 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300, 1005-1016.

Farré, E.M., Tiessen, A., Roessner, U., Geigenberger, P., Trethewey, R.N., Willmitzer, L.,. 2001. Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. Plant Physiol. 127, 685-700.

Fox, T.C., Green, B.J., Kennedy, R.A., Rumpho, M.E., 1998. Changes in hexokinase activity in *Echinochloa phyllopogon* and *Echinochloa crus-pavonis* in response to abiotic stress. Plant Physiol. 118, 1403-1409.

Frömmer, W.B., Schulze, W.X., Lalonde, S., 2003. Hexokinase, Jack-of-all-trades. Science 300, 261-263.

Galina, A., Logullo, C., de Souza, E.F., Rezende, G.L., da-Silva, W.S., 1999. Sugar phosphorylation modulates ADP inhibition of maize mitochondrial hexokinase. Physiol. Plant. 105, 17-23.

Galina, A., Reis, M., Albuquerque, M.C., Puyou, A.G., Puyou, M.T., de Meis, L., 1995. Different properties of the mitochondrial and cytosolic hexokinases in maize roots. Biochem. J. 309, 105-112.

Gerhardt, R., Stitt, M., Heldt, H.W., 1987. Subcellular metabolite levels in spinach leaves: Regulation of sucrose synthesis during diurnal alterations in photosynthetic partitioning. Plant Physiol. 83, 399-407.

Giegé, P., Heazlewood, J.L., Roessner-Tunali, U., Millar, A.H., Fernie, A.R., Leaver, C.J., Sweetlove, L.J., 2003. Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. Plant Cell 15, 2140-2151.

Giese, J.O., Herbers, K., Hoffmann, M., Klosgen, R.B., Sonnewald, U., 2005. Isolation and functional characterization of a novel plastidic hexokinase from *Nicotiana tabacum*. FEBS Lett. 579, 827-831.

Gonzali, S., Alpi, A., Blando, F., de Bellis, L., 2002. *Arabidopsis* (HXK1 and HXK2) and yeast (HXK2) hexokinases overexpressed in transgenic lines are characterized by different catalytic properties. Plant Sci. 163, 943-954.

Guglielminetti, L., Perata, P., Morita, A., Loreti, E., Yamaguchi, J., Alpi, A., 2000. Characterization of isoforms of hexose kinases in rice embryo. Phytochem. 53, 195-200.

Harrington, G.N., Bush, D.R., 2003. The bifunctional role of hexokinase in metabolism and glucose signaling. Plant Cell 15, 2493-2496.

Higgins, T.J., Easterby, J.S., 1974. Wheat germ hexokinase: physical and active-site properties. Eur. J. Biochem. 45, 147-160.

Higy, M., Junne, T., Spiess, M., 2004. Topogenesis of membrane proteins at the endoplasmic reticulum. Biochemistry 43, 12716-12722.

Hooks, M.A., Shearer, G.C., Roberts, J.K.M., 1994. Nucleotide availability in maize (*Zea mays* L.) root tips (estimation of free and protein-bound nucleotides using ^{31}P-nuclear magnetic resonance and a novel protein-ligand-binding assay). Plant Physiol. 104, 581-589.

Jang, J.C., Leon, P., Zhou, L., Sheen, J., 1997. Hexokinase as a sugar sensor in higher plants. Plant Cell 9, 5-19.

Jang, J.C., Sheen, J., 1994. Sugar sensing in higher plants. Plant Cell 6, 1665-1679.

Koch, K.E., 1996. Carbohydrate-modulated gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 509-540.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lalonde, S., Wipf, D., Frömmer, W.B., 2004. Transport mechanisms for inorganic forms of carbon and nitrogen between source and sink. Annu. Rev. Plant Biol. 55, 341-372.

Lantin, S., O'Brien, M., Matton, D.P., 1999. Pollination, wounding and jasmonate treatments induce the expression of a developmentally regulated pistil dioxygenase at a distance, in the ovary, in the wild potato *Solanum chacoense* Bitt. Plant Mol. Biol. 41, 371-386.

Martinez-Barajas, E., Randall, D.D., 1998. Purification and characterization of a glucokinase from young tomato (*Lycopersicon esculentum* L. Mill.) fruit. Planta 205, 567-573.

Menu, T., Rothan, C., Dai, N., Petreikov, M., Etienne, C., Destrac-Irvine, A., Schaffer, A., Granot, D., Ricard, B., 2001. Cloning and characterization of a cDNA encoding hexokinase from tomato. Plant Sci. 160, 209-218.

Miernyk, J.A., Dennis, D.T., 1983. Mitochondrial, plastid, and cytosolic isozymes of hexokinase from developing endosperm of *Ricinus communis*. Arch. Biochem. Biophys. 226, 458-468.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Liu, Y.X., Hwang, I., Jones, T., Sheen, J., 2003. Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science 300, 332-336.

Nakai, K., Kanehisa, M., 1991. Expert system for predicting protein localization sites in gram-negative bacteria. Proteins 11, 95-110.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1-6.

Olsson, T., Thelander, M., Ronne, H., 2003. A novel type of chloroplast stromal hexokinase is the major glucose-phosphorylating enzyme in the moss *Physcomitrella patens*. J. Biol. Chem. 278, 44439-44447.

Paul, M., Pellny, T., Goddijn, O., 2001. Enhancing photosynthesis with sugar signals. Trends Plant Sci. 6, 197-200.

Pego, J.V., Smeekens, S., 2000. Plant fructokinases: a sweet family get-together. Trends Plant Sci. 5, 531-536.

Plaxton, W.C., 1989. Molecular and immunological characterization of plastid and cytosolic pyruvate kinase isozymes from castor-oil-plant endosperm and leaf. Eur. J. Biochem. 181, 443-451.

Plaxton, W.C., 1996. The organization and regulation of plant glycolysis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 185-214.

Renz, A., Merlo, L., Stitt, M., 1993. Partial purification from potato tubers of three fructokinases and three hexokinases which show differing organ and developmental specificity. Planta 190, 156-165.

Renz, A., Stitt, M., 1993. Substrate specificity and product inhibition of different forms of fructokinases and hexokinases in developing potato tubers. Planta 190, 166-175.

Rivoal, J., Trzos, S., Gage, D.A., Plaxton, W.C., Turpin, D.H., 2001. Two unrelated phospho*enol*pyruvate carboxylase polypeptides physically interact in the high molecular mass isoforms of this enzyme in the unicellular green alga *Selenastrum minutum*. J. Biol. Chem. 276, 12588-12597.

Roberts, J.K., Callis, J., Wemmer, D., Walbot, V., Jardetzky, O., 1984. Mechanisms of cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under hypoxia. Proc. Natl. Acad. Sci. USA 81, 3379-3383.

Rolland, F., Moore. B., Sheen, J., 2002. Sugar sensing and signaling in plants. Plant Cell 14 (Suppl.), S185-S205.

Rolland, F., Winderickx, J., Thevelein, J.M., 2001. Glucose-sensing mechanisms in eukaryotic cells. Trends Biochem. Sci. 26, 310-317.

Salas, J., Salas, M., Vinuela, E., Sols, A., 1965. Glucokinase of rabbit liver. Purification and properties. J. Biol. Chem. 240, 1014-1018.

Schluepmann, H., Pellny, T., van Dijken, A., Smeekens, S., Paul, M., 2003. Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 100, 6849-6854.

Schnarrenberger, C., 1990. Characterization and compartmentation, in green leaves, of hexokinases with different specificities for glucose, fructose, and mannose and for. nucleoside triphosphates. Planta 181, 249-255.

Sheen, J., Zhou, L., Jang, J.C., 1999. Sugars as signalling molecules. Curr. Opin. Plant Biol. 2, 410-418.

Stoscheck, C.M., 1990. Quantitation of protein. Methods Enzymol. 182, 50-68.

Thevelein, J.M., Hohmann, S., 1995. Trehalose synthase: guard to the gate of glycolysis in yeast? Trends Biochem. Sci. 20, 3-10.

Turner, J.F., Copeland, L., 1981. Hexokinase II of pea seeds. Plant Physiol. 68, 1123-1127.

van Dongen, J.T., Roeb, G.W., Dautzenberg, M., Froehlich, A., Vigeolas, H., Minchin, P.E.H., Geigenberger, P., 2004. Phloem import and storage metabolism are highly coordinated by the low oxygen concentrations within developing wheat seeds. Plant Physiol. 135, 1809-1821.

Veramendi, J., Roessner, U., Renz, A., Willmitzer, L., Trethewey, R.N., 1999. Antisense repression of hexokinase 1 leads to an overaccumulation of starch in leaves of transgenic potato plants but not to significant changes in tuber carbohydrate metabolism. Plant Physiol. 121, 123-134.

Wiese, A., Groner, F., Sonnewald, U., Deppner, H., Lerchl, J., Hebbeker, U., Flügge, U., Weber, A., 1999. Spinach hexokinase I is located in the outer envelope membrane of plastids. FEBS Lett. 461, 13-18.

Wray, W., Boulikas, T., Wray, V.P., Hancock, R., 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. 118, 197-203.

Yamamoto, Y.T., Prata, R.T.N., Williamson, J.D., Weddington, M., Pharr, D.M., 2000. Formation of a hexokinase complex is associated with changes in energy utilization in celery organs and cells. Physiol. Plant. 110, 28-37.

Chapitre 4.

Modifier les Niveaux d'Activité Hexokinase de Racines Transgéniques de Pomme de Terre (*Solanum tuberosum*) Altère leur Croissance et le Flux Glycolytique à l'Étape de la Phosphorylation du Glucose

Claeyssen, Dorion, Wally, Clendenning et Rivoal, 2007. Soumis à J. Biol. Chem.

Contribution des coauteurs :

Je suis l'auteur principal du texte de ce manuscrit, pour lequel j'ai monté les figures et le Tableau 4.1. Les clones racinaires à l'origine de cette étude ont été générés par O. Wally. Les dosages de métabolites par HPLC et spectrométrie ont été menés par la Dre. S. Dorion (Figs. 4.4–4.7; Tableau 4.2). A. Clendenning a contribué aux extractions de métabolites pour les Figures 4.4 à 4.6 et le Tableau 4.2. J'ai réalisé les autres expériences.

4.1 Title page

Altering hexokinase activity levels in transgenic potato (*Solanum tuberosum*) roots affects their growth and glycolytic flux at the step of glucose phosphorylation*

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*This work was supported by a Discovery Grant from the National Science and Engineering Research Council of Canada to J. R.

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Submitted to Journal of Biological Chemistry 8 May 2007.

4.2 Preliminary material

4.2.1 Abbreviations

The abbreviations used were: α-KG, α-ketoglutarate; DOG, 2-deoxy-D-glucose; DOG6P, 2-deoxy-D-glucose-6-phosphate; F6P, D-fructose-6-phosphate; FCC, flux control coefficient; FK, fructokinase; Fru, D-fructose; G1P, D-glucose-1-phosphate; G6P, D-glucose-6-phosphate; GABA, γ-aminobutyric acid; GK, glucokinase; GKRP: GK-regulatory protein; Glc, D-glucose; Glc-P, G1P or G6P; GOGAT, Glu synthase; GS, Gln synthetase; hexose-P, hexose-phosphate; HK, hexokinase; INV, invertase; MYA, mannitol/yeast extract/ammonium sulfate; OAA, oxaloacetate; OPPP, oxidative pentose-phosphate pathway; PEP, phospho*enol*pyruvate; PEPC, PEP carboxylase; Suc, D-sucrose; SuSy, D-sucrose synthase; T6P, D-trehalose-6-phosphate; TCA, tricarboxylic acid; U, unit.

4.3 Abstract

We have examined the effects of altering hexokinase (HK, EC 2.7.1.1) activity of plant roots on their growth and metabolism. Potato (*Solanum tuberosum*) roots were transformed with a *Solanum chacoense* HK cDNA in sense and antisense orientations to constitutively over- and underexpress HK, respectively. Twenty-three root clones were generated that exhibited more than an 11-fold variation in HK activity levels (between 72% and 800% of those in control clones transformed with an empty vector). Protein separation by anion-exchange chromatography revealed at least two HK isoforms in the potato roots, which activities were differently affected by sense and antisense strategies. A survey of 18 enzymes of primary metabolism showed that only HK activity was modified significantly in the transgenics. Raising their HK activity levels tended to limit their growth, thus showing for the first time that root HK exerts major control on growth of this heterotrophic organ. We observed a trend towards a proportionate decrease in D-glucose and D-fructose contents with increasing HK activities in the transgenic population, an indication that both sugars may serve as substrates for the HK reaction. Levels of malate, Arg, Phe, Thr and γ-aminobutyric acid also varied at the opposite of HK activities, which may be best explained

in terms of interference of HK manipulation with their biosynthesis. The flux control coefficient of HK over glycolysis at the step of D-glucose phosphorylation was measured for the first time in plants, and was particularly high (0.76). The high flux control coefficient of HK and the metabolic perturbations occurring in the transgenic roots are discussed in relation to the effects of altered HK activity on their growth behavior.

4.4 Introduction

Glycolysis is central to cell metabolism as it oxidizes hexoses to generate ATP and produces building blocks for various biosynthetic pathways (Plaxton, 1996). Hexokinase (HK, EC 2.7.1.1) is a glycolytic enzyme that catalyzes the irreversible, ATP-dependent phosphorylation of hexoses such as D-glucose (Glc) and D-fructose (Fru). Its specificity for several hexose substrates makes this enzyme a gateway to carbohydrate metabolism, and a potentially major controlling step of the glycolytic pathway (Renz and Stitt, 1993; Wilson, 2003; Claeyssen and Rivoal, 2007). Accordingly, HK has been identified as an important site of glycolytic flux control in several eukaryotic organisms, based on metabolic control analysis (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). Thus, the flux control coefficient (FCC) of HK over glycolysis exceeds 0.7 in mammalian erythrocytes, liver, heart, pancreas, insulinoma and muscle cells (Rapoport *et al.*, 1974; Meléndez-Hevia *et al.*, 1992; Kashiwaya *et al.*, 1994; Sweet and Matschinsky, 1995; Wang and Iynedjian, 1997; Puigjaner *et al.*, 1997). In yeast (*Saccharomyces cerevisiae*), mutant studies have implicated HK in the control of glycolytic flux via its inhibition by D-trehalose-6-phosphate (T6P) (Thevelein and Hohmann, 1995; Teusink *et al.*, 1998). It has been proposed that yeast glycolysis operates according to an autocatalytic principle in which ATP is consumed to drive Glc catabolism before being replenished by subsequent metabolism. HK inhibition by T6P would restrict Glc influx into glycolysis, thereby preventing a stall during an abrupt increase in Glc supply (Teusink *et al.*, 1998). Accordingly, there is evidence to suggest that the relatively low (0.2 to 0.5) FCC of HK over glycolysis may be due to its potent inhibition by T6P in yeast (Aon and Cortassa, 1998; Ernandes *et al.*, 1998). With respect to plants, the FCC of HK over glycolysis still awaits measurement and the question of

whether and how HK may control glycolytic flux is still ill-defined (Claeyssen and Rivoal, 2007). Nonetheless, indirect evidence suggests an important role for HK in the regulation of primary metabolism. Thus, increasing HK activity levels in tomato (*Solanum lycopersicum*) plants resulted in growth inhibition and perturbed carbon metabolism (Dai *et al.*, 1999; Menu *et al.*, 2004). HK, also, is likely to take part in D-sucrose (Suc) cycles and in cycling between Glc and Glc-P, i.e. D-glucose-1-phosphate (G1P) or D-glucose-6-phosphate (G6P) (Nguyen-Quoc and Foyer, 2001; Alonso *et al.*, 2005). Altogether, the Suc and Glc/Glc-P substrate ('futile') cycles may consume 40–80% of the ATP generated in maize (*Zea mays*) root tips (Dieuaide-Noubhani *et al.*, 1995; Rontein *et al.*, 2002; Alonso *et al.*, 2005). Interestingly, it has been shown that plant glycolytic flux is controlled, at least in part, by ATP demand (Fernie *et al.*, 2002; Sweetlove *et al.*, 2002). Suc and Glc/Glc-P cycles, therefore, have been proposed to raise glycolytic flux through increased energy demand (Fernie *et al.*, 2002; Alonso *et al.*, 2005). Altogether, these findings suggest new possibilities for plant HK to contribute to glycolytic flux control via Suc and Glc/Glc-P cycling and modulation of the cell's energy status (Claeyssen and Rivoal, 2007).

Apart from its metabolic role, HK functions as a sugar sensor in Eukaryotes (Rolland *et al.*, 2001; Frömmer *et al.*, 2003). Sugars act in plants as signals that control growth and development throughout the life cycle (Rolland *et al.*, 2006). It has been unambiguously demonstrated that the function of HK in sugar sensing and signaling is distinct and independent from its catalytic activity (Moore *et al.*, 2003). Indeed, Glc responses in growth and gene expression were restored in an *Arabidopsis* mutant mutated in the HK gene *HXK1* when transformed with engineered HXK1 proteins that lacked catalytic activity but still mediated Glc signaling (Moore *et al.*, 2003). This new function in hexose sensing and signaling has been put forward to explain the impact of HK overexpression on growth, photosynthetic activity and senescence of tomato green tissues (Dai *et al.*, 1999).

Plant roots depend on the hexose supply from other plant parts for their carbon metabolism. In that view, we aimed at clarifying the relevance of HK to primary metabolism and to the control of glycolytic flux in this heterotrophic organ. Potato (*Solanum tuberosum*) roots were altered in their HK activities by transformation with an

HK cDNA from the wild relative *Solanum chacoense* (Claeyssen *et al.*, 2006). Raising their HK activity levels tended to limit their growth, thus showing the importance of the catalytic function of HK in root growth regulation. The clone population was also used to determine the FCC of HK over glycolytic flux at the step of Glc phosphorylation. The FCC was found at the considerably high value of 0.76, which strongly suggests that HK exerts tight control on the upper part of glycolysis in plants. Based on these data, we propose that the tight control of root HK on flux at the Glc phosphorylation step has major implications in the regulation of root growth.

4.5 Experimental procedures

4.5.1 Materials and chemicals

All buffers, chemicals, reagents and commercial enzymes were of analytical grade and purchased from Sigma Chemical Co. (St-Louis, MO) or Fisher Scientific (Nepean, ON, Canada), unless otherwise mentioned. PD10 columns were from G.E. Healthcare (Baie d'Urfé, QC, Canada). Fractogel EMD DEAE-650 (S) was from VWR (Mississauga, ON, Canada). Dowex AG 50W-X8 (H^+) and Dowex AG 1-X8 (formate) resins were from Bio-Rad Laboratories Inc. (Mississauga, ON, Canada). Restriction enzymes were from MBI-Fermentas and Invitrogen Canada Inc. (Burlington, ON, Canada). Radiolabeled tracers 2-deoxy-D-[U-^{14}C]glucose ([U-^{14}C]DOG) and 2-deoxy-D-[1-^{3}H]glucose ([1-^{3}H]DOG) were purchased from Moravek Biochemicals Inc. (Brea, CA, USA).

4.5.2 Plant material, bacterial strains, and plasmids

Potato plants (*Solanum tuberosum*, cv. Russet Burbank) used for transformation were grown in growth chambers at 23 °C with 12 h light period until the age of 4 to 6 weeks. *Agrobacterium rhizogenes* wild-type strain A4 (Moore *et al.*, 1979) was obtained from Dr. D. Tepfer (Institut National de la Recherche Agronomique, Versailles, France) and was maintained on mannitol/yeast extract/ammonium sulfate (MYA) medium (Petit *et*

al., 1983). *Escherichia coli* HB101 carrying the plant expression plasmid pGA643 (An *et al.*, 1988) was maintained on Luria–Bertani broth medium (Sambrook and Russell, 2001) containing 10 mg L^{-1} kanamycin and 2 mg L^{-1} tetracycline. *A. rhizogenes* A4 carrying pGA643 was grown on MYA medium containing 25 mg L^{-1} kanamycin and 5 mg L^{-1} tetracycline.

4.5.3 Construction of plasmids

Cloning, recombinant DNA work and DNA analyses were done according to established techniques (Sambrook and Russell, 2001). One sense and three antisense constructs were prepared using *ScHK2*, a previously characterized HK cDNA from *Solanum chacoense* (GenBank Accession No. DQ177440) (Claeyssen *et al.*, 2006). This sequence shares over 97% homology with potato StHK2 (Claeyssen *et al.*, 2006) and was therefore expected to be used successfully in an antisense strategy. Sense and antisense *ScHK2* constructs were inserted into the binary vector pGA643 under the control of the cauliflower mosaic virus 35S promoter, using the neomycin phosphotransferase II (*npt*-II) gene as a selectable marker (An *et al.*, 1988). The sense construct was obtained by digesting the *ScHK2* cDNA from pBK-CMV (Claeyssen *et al.*, 2006) with *Cla*I and *Hinc*II. The resulting 1850 bp fragment was cloned into *Cla*I:*Hpa*I-digested pGA643 plasmid. Digestion of the *ScHK2* cDNA with *Xba*I and *Eco*RV, and with *Xba*I and *Hinc*II, generated antisense constructs 1 (606 bp) and 3 (1829 bp), respectively. Each construct was cloned into *Xba*I:*Hpa*I-digested pGA643 plasmid. For preparation of antisense construct 2, pGA643 was digested with *Hpa*I and dephosphorylated with 150 U of bacterial alkaline phosphatase (Invitrogen) for 1 h at 65 °C. Bacterial alkaline phosphatase was removed according to manufacturer's instructions. The *ScHK2* cDNA was then digested with *Eco*RV and *Hinc*II into a 1212 bp fragment that was cloned into prepared pGA643 vector. Presence and correct orientation of the inserts were confirmed by restriction digests. Root clones transformed with an empty vector were named Ctrl00, whereas sense clones were named S100. Antisense clones transformed with constructs 1, 2 and 3 were named AS100, AS200 and AS300, respectively. The last two digits identify individual clones.

4.5.4 Stem transformation and root cultures

Freshly cut potato stems were transformed as described by Rivoal and Hanson (1994) for tomato petioles, except that Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) was supplemented here with 3% (w/v) Suc. Solid MS medium was obtained with 0.4% (w/v) Phytagel. Roots were routinely subcultured every 3 weeks on solid MS medium. Root clones were screened based on Southern, immunodetection and HK activity analyses that are described below.

4.5.5 Southern analysis

DNA extraction from frozen roots was adapted from Joly and Bruneau (2006). DNA extracts were digested with *Xba*I, *Hin*dIII, or *Eco*RV, separated, and transferred onto neutral nylon blots (Mandel, Guelph, ON, Canada). Two different probes were used for hybridization. The region of pGA643 vector encompassing the *npt*-II gene and CAMV 35S promoter sequences was used as template for one probe. The full-length *ScHK2* cDNA served as template for the second probe. Both probes were synthesized using the HexaLabel Plus DNA labeling kit and [α-^{32}P]ATP (MBI-Fermentas). After overnight hybridization at 65 °C, Southern blots were washed with a graded series from 2x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) in 0.1% (w/v) SDS at 40 °C to 0.1x SSC at 55 °C. Hybridizing bands were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

4.5.6 HK extraction from potato roots and activity assay

Root clones were subcultured in 250 mL Erlenmeyer flasks containing 25 mL of liquid MS medium and agitated continuously at 145 rpm on a gyratory shaker, at 23 °C. Roots were harvested after 6 to 34 days of growth in liquid MS medium, snap frozen with liquid nitrogen and stored at –80 °C until used. All subsequent steps were carried out at 4 °C. Roots were ground with a mortar and pestle using a 3:1 (mL extraction buffer g FW^{-1}) ratio in a buffer containing 100 mM Tris-HCl, pH 8.0, 10 mM KCl, 10 mM $MgCl_2$, 5 mM

DTT, 1 mM EDTA, 1 mM EGTA, 1 mM ε-amino-N-caproic acid (ε-CA), 1 mM benzamidine, 5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP), 0.1% (v/v) Triton X-100, 10% (v/v) glycerol and 1 mM phenylmethylsulfonylfluoride (PMSF) added fresh. Homogenates were centrifuged for 15 min at 12,000 x *g*. This step was repeated in case of debris remaining in supernatants. The resulting supernatants were desalted on PD10 columns pre-equilibrated with desalting buffer (20 mM Tris-HCl, pH 8.2, 0.5 mM $MgCl_2$, 1 mM DTT and 10% [v/v] glycerol). HK activity assays were conducted on desalted extracts as in (Claeyssen *et al.*, 2006). One unit (U) of HK activity corresponds to the appearance of G6P at the rate of 1 µmol min^{-1} at 30 °C. Protein concentration was determined as indicated below.

4.5.7 Extraction and assays of other primary metabolism enzymes

All steps were carried out at 4 °C. Roots were ground with a mortar and pestle using a 2:1 (mL extraction buffer g FW^{-1}) ratio in a buffer containing 30 mM Hepes-KOH, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 1 mM EGTA, 5 mM ε-CA, 1 mM benzamidine, 1 mg L^{-1} leupeptin, 5% (w/v) insoluble PVPP, 0.1% (v/v) Triton X-100, 4% (w/v) polyethylene glycol 8000, 20% (v/v) glycerol and 2 mM PMSF. Homogenates were centrifuged for 15 min at 12,000 x *g*. Clear supernatants were desalted on PD10 columns pre-equilibrated with desalting buffer (30 mM Hepes-KOH, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 4% [w/v] polyethylene glycol and 20% [v/v] glycerol). The following enzymes were measured using coupled assays based on production or consumption of NADH at 340 nm, at 30 °C. Phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) was assayed as in (Moraes and Plaxton, 2000). Assays of pyruvate kinase (PK, EC 2.7.1.40) and phospho*enol*pyruvate phosphatase (PEPase, EC 3.1.3.60) were modified from Duff *et al.* (1989) and Plaxton *et al.* (2002). Assays of phosphoglucomutase (PGM, EC 5.4.2.2), D-sucrose synthase (SuSy, EC 2.4.1.13), ADPglucose pyrophosphorylase (AGPase, EC 2.7.7.27) and UDPglucose pyrophosphorylase (UGPase, EC 2.7.7.9) were adapted from Sweetlove *et al.* (1996). Phosphoglucose isomerase (PGI, EC 5.3.1.9) assay was modified from Rivoal *et al.* (1989). Aldolase (ALD, EC 4.1.2.13) was assayed as in (Hodgson and Plaxton, 1998). Assays for

ATP-dependent phosphofructokinase (PFK, EC 2.7.1.11), D-fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) and pyrophosphate-D-fructose-6-phosphate 1-phosphotransferase (PFP, EC 2.7.1.90) were modified from Wood *et al.* (2002). Assay for triosephosphate isomerase (TPI, EC 5.3.1.1) was from Dorion *et al.* (2005). Assay of NAD(P)-dependent D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12 and EC 1.2.1.13) was modified from Plaxton (1990). Assays were initiated by addition of enzyme preparation and corrected for background activity by omitting substrate from the reaction mixture. Invertases (INVs) were assayed by quantification of reducing sugars at 525 nm against a standard curve of Glc established in the same conditions. Soluble (acid, pH 4.8, and neutral, pH 7.0) INVs were assayed in desalted extracts and cell-wall INV (pH 4.8) in pellets after centrifugation, according to a protocol adapted from Sung *et al.* (1989), Dorion *et al.* (1996) and Appeldoorn *et al.* (1997). Protein levels were adjusted to keep any potential inhibitory effect on INVs (Sander *et al.*, 1996) constant among samples. For all enzymes studied, reaction rates were linear with time and proportional to the amount of enzyme added to the assay. The stoichiometry of the reactions was taken in account in activity calculations. Specific activities are expressed in U mg protein^{-1} (1 U of activity corresponds to the appearance of reaction product at the rate of 1 µmol min^{-1} at 30 °C). Protein concentration was determined as indicated below.

4.5.8 SDS–PAGE, immunoblot analysis of HK and protein determination

SDS–PAGE analysis was performed on 12% acrylamide gels according to Laemmli (1970). Aliquots of desalted extracts were heat denatured in SDS sample buffer and frozen at –20 °C until used. Immunodetection analysis was performed with affinity-purified rabbit immune serum (1/15 dilution) raised against a truncated version of the ScHK2 protein (Claeyssen *et al.*, 2006). Immunoblots incubated with the pre-immune serum gave negative results (data not shown). Protein concentration was determined according to Bradford (1976), using BSA as standard and the Bio-Rad protein assay reagent (Bio-Rad Laboratories Inc.).

4.5.9 Analytical anion-exchange chromatography of HK isoforms

Roots grown on solid MS medium for 20 to 26 days were extracted and clarified as described above for HK activity assays except that extraction buffer contained 50 mM Tris-HCl, pH 8.0, and 5 mM $MgCl_2$. Clarified extracts were then desalted and loaded onto a column (1 x 8 cm) of Fractogel EMD DEAE-650 (S) as described in (Claeyssen *et al.*, 2006). One-mL fractions were collected and assayed. Activity recovery from the column reached 58% and 77% of glucokinase (GK) and fructokinase (FK) activities loaded, respectively. Typically, HK activity was resolved as two peaks eluting between 125 and 195 mM KCl. An additional FK activity peak eluted between 235 and 300 mM KCl. Representative activity profiles are shown.

4.5.10 Growth measurement

Root tips, 0.6 to 0.8 cm in length, were laid in 52 x 15-mm Petri plates containing 15 mL of solid MS medium supplemented with 0.2% Phytagel, and left to grow in the dark. Significant differences in growth patterns of the root clones were observed during the logarithmic phase, which was reached on day 10 of culture. Therefore, roots were scanned on day 14 of culture and analyzed with the WinRhizo software (Régent Instruments Inc., Québec, QC, Canada) for measurement of total root length, tip number and mean diameter.

4.5.11 Synthesis of radio-labeled standard 2-deoxy-D-[1-^{3}H]glucose-6-phosphate

2-deoxy-D-[1-^{3}H]glucose-6-phosphate ([1-^{3}H]DOG6P) was prepared from [1-^{3}H]DOG, and used as internal standard for monitoring the recovery of [U-^{14}C]DOG6P from extraction procedures. Twenty µCi of [1-^{3}H]DOG (4 Ci mmol^{-1}) were added to 100 µL buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 20 mM $MgCl_2$, 6.5 mM DOG, 20 mM ATP and 0.18 U of commercial yeast HK. The mixture was incubated for 30 min at 37 °C, and brought to a 3-mL final volume containing 1 µmol DOG and 1 µmol DOG6P (carriers). The solution was fractionated on Dowex AG 50W-X8 (H^+) resin (counter-ion: NH_4^+), then on Dowex AG 1-X8 (formate) resin (counter-ion: formate), as in (Canvin and

Beevers, 1961). [1-^{3}H]DOG6P eluted in the acidic fraction, which was vacuum dried for 4 h and re-suspended in 5% ethanol. [1-^{3}H]DOG6P was counted using a scintillation counter, and the final volume was adjusted so that 20 µL of solution contained 100,000 cpm of [1-^{3}H]DOG6P.

4.5.12 Labeling of roots with 2-deoxy-D-[U-^{14}C]glucose

Roots grown in liquid MS medium for 3 to 10 days were transferred to modified MS medium containing 0.02% Suc for 17 h prior to labeling with [U-^{14}C]DOG. This pre-treatment allowed for sustained metabolism of the tracer. Accumulation rates of [U-^{14}C]DOG6P were linear over time between 0 and 6 h of labeling (data not shown), indicating that the tissue was at metabolic and isotopic steady state within this time frame (Salon *et al.*, 1988). [U-^{14}C]DOG6P declined thereafter, most probably as a result of its catabolism, as described elsewhere (Klein and Stitt, 1998). Consequently, [U-^{14}C]DOG6P levels were assessed after 2 and 8 h of labeling to measure glycolytic flux at steady state and to investigate [U-^{14}C]DOG6P catabolism, respectively. Roots (0.25 g FW) were incubated in 0.5 µCi of [U-^{14}C]DOG (230-330 mCi mmol^{-1}) for 2 or 8 h, then stored overnight at –20 °C in 10 mL of 80% ethanol in the presence of 1 µmol DOG and 1 µmol DOG6P added as carriers. After evaporation of ethanol at 75 °C, extracts were re-solubilized in 3 mL of sterile H_2O. Extracts were then fractionated on columns of Dowex AG 50W-X8 (H^+) resin, and Dowex AG 1-X8 (formate) resin arranged in tandem. [U-^{14}C]DOG and [U-^{14}C]DOG6P eluted in neutral and acidic fractions, respectively, and were counted with a scintillation counter. Aliquots of 1.2 mL were frozen at –80 °C for subsequent analysis by HPLC. Losses of [U-^{14}C]DOG6P during extraction were estimated using [1-^{3}H]DOG6P as an internal standard. A spike (100,000 c.p.m.) of [1-^{3}H]DOG6P was added to every sample during fixation in 80% ethanol. Losses of [U-^{14}C]DOG6P were corrected for every sample considering that losses of [U-^{14}C]DOG6P and [1-^{3}H]DOG6P were comparable during extraction.

4.5.13 Metabolite measurements

Roots were grown in liquid MS medium for 4 days, medium was then replenished for another 2 days of growth. Roots were then stored at –80 °C after flash freezing with liquid nitrogen. For the spectrophotometric determination of hexose-phosphates (hexose-Ps), roots were processed and analyzed as described by Gauthier and Turpin (1994), and data were corrected for recovery. For HPLC analysis, a precisely-weighed amount of approximately 0.5 g FW frozen material was placed in 10 mL of 80% ethanol in the presence of 4 µmol mannitol (internal standard). After evaporation of ethanol at 75 °C, extracts were re-solubilized in 3 mL of sterile H_2O and fractionated on Dowex AG 50W-X8 (H^+) resin (counter-ion: NH_4^+) then on Dowex AG 1-X8 (formate) resin (counter-ion: formate). Sugars, organic acids and amino acids eluted in neutral, acidic and basic fractions, respectively, and were analyzed by HPLC as described by Dorion *et al.* (in preparation).

4.5.14 Statistical analyses

Wilcoxon's rank sum test was performed using the algorithm embedded into S-Plus 7.0 (Insightful Corporation, Seattle, WA, USA). The Pearson correlation coefficient was calculated using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

4.6 Results and discussion

4.6.1 Generation and characterization of 26 root clones with differing levels of HK expression and activity

We generated a population of root clones with varying HK activity levels to study the control of HK on root growth and on glycolytic flux at the step of Glc phosphorylation. One sense and three antisense constructs of the *ScHK2* cDNA (Claeyssen *et al.*, 2006), inserted in the pGA643 vector under the control of the CAMV 35S promoter, were used for *A. rhizogenes*-mediated transformation of potato stems. Depending on the HK construct

introduced, the root clones constitutively over- or underexpressed HK. The advantage of introducing *ScHK2* in the HK-overexpressing roots was that it shared over 97% homology with its counterpart StHK2 from *S. tuberosum* (Claeyssen *et al.*, 2006). Consequently, the novel HK activity was expected to be efficiently regulated in the host roots. Twenty-six clones were generated from independent transformation events, as shown by unique hybridization patterns in Southern analysis using genomic DNA restricted with *Xba*I, *Hin*dIII, or *Eco*RV, and two radioactive probes (data not shown). Three control clones transformed with an empty pGA643 vector served as references for screening of the clone population based on *in vitro* HK activity levels (Fig. 4.1a,b). Control clones had a mean specific HK activity of 18.4 ± 0.95 mU mg protein^{-1} (Fig. 4.1a). Thirteen antisense clones were generated that displayed between 72% and 90% of HK activity levels observed for the control clones. Transgenics displaying lower activity levels could not be found in the screening despite the use of 3 different cDNA constructs in the antisense strategy. Ten sense clones had HK activity levels 1.5 to 8 times higher than those of control clones (Fig. 4.1a). GK and FK components of these HK activities were well correlated, and the order of clones was almost identical between GK and FK activity levels (Fig. 4.1a,b). The 26 clones were subjected to immunoblot analysis with affinity-purified immune serum (Claeyssen *et al.*, 2006), as shown in Fig. 4.1c. The rise in HK activity levels among the clones was broadly consistent with an increase in HK protein levels (Fig. 4.1a,b,c).

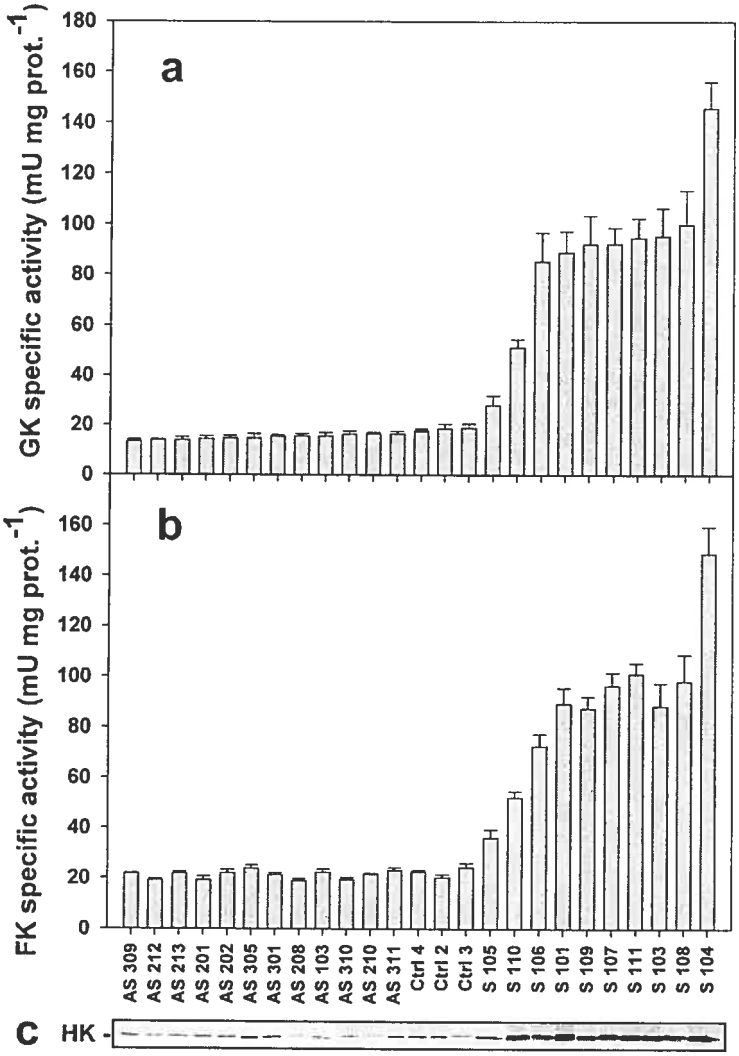


Figure 4.1: Total extractable HK activity and protein levels in potato root clones constitutively over- or underexpressing HK, or transformed with an empty pGA643 vector.

a–b, GK and FK components of HK specific activity levels in antisense (AS) and sense (S) clones, and in control (Ctrl) clones containing an empty pGA643 vector. Values are means ± SE from three to ten separate extractions. c, Representative immunoblot analysis of HK steady-state levels in potato root clones. Protein extracts were separated by SDS–PAGE and transferred onto a PVDF membrane. Immunodetection was carried out with affinity-purified immune serum raised against a truncated version of ScHK2 (HK). Each lane was loaded with 6.5 μg of protein.

4.6.2 HK isoform profiles of three representative potato root clones

Multiple HK and FK isoforms have been described in potato tubers and leaves, and in many other systems (Renz *et al.*, 1993; Claeyssen and Rivoal, 2007). Here, the hexose-phosphorylating activities present in potato roots were examined by analytical anion-exchange chromatography (Fig. 4.2). Protein extracts from representative antisense (AS301), control (Ctrl3) and sense (S111) clones were separated by anion-exchange chromatography to study the impact of sense and antisense strategies on the potato root HK and FK isoform profiles. Two peaks, identified 1 and 2, eluted respectively at fractions 20 and 23 in the GK (Fig. 4.2b) and FK (Fig. 4.2e) profiles of clone Ctrl3, indicating the existence of at least two HK isoforms in the roots of *S. tuberosum*. A third peak (peak 3) with only FK activity eluted between fractions 30 and 43 (Fig. 4.2e). Peak 1 represented 65% of total GK activity, whereas peak 2 represented 35% of total GK activity (data not shown). With respect to clone AS301, peaks 1 and 2 were observed at the same fractions (20–21 and 24) (Fig. 4.2a,b,d,e), and accounted for 52% and 48% of total GK activity, respectively (data not shown). It may then be proposed that the antisense strategy affected most particularly the HK isoform(s) corresponding to peak 1 of activity. In clone S111 a single, large peak of HK activity eluted at fraction 23 and hence, was also named peak 2 (Fig. 4.2c,f). Possibly, ScHK2 was strongly expressed in the S111 clone, and gave rise to a large activity peak engulfing the two preexisting peaks 1 and 2 (Fig. 4.2b,c,e,f).

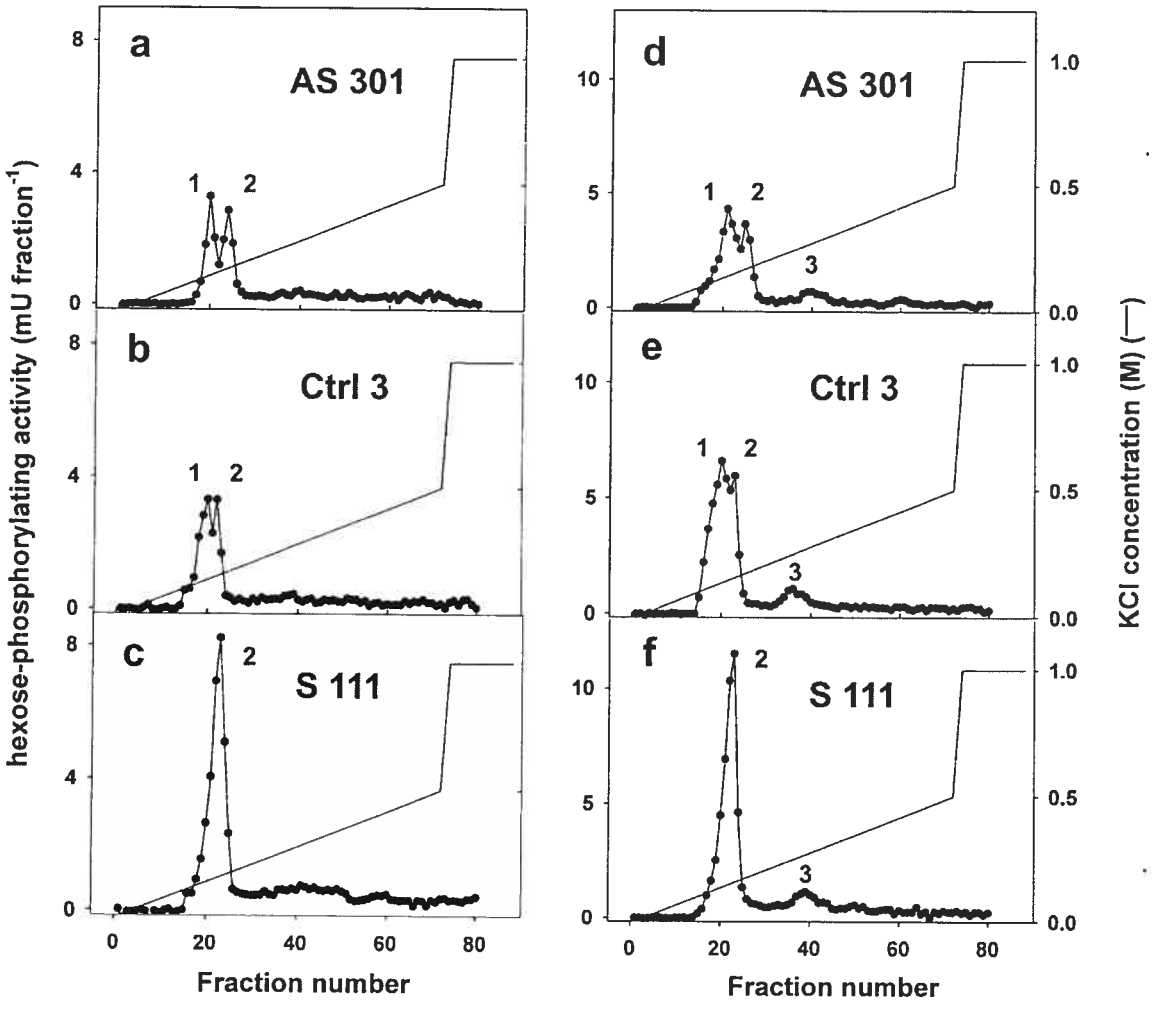


Figure 4.2: Analytical separation of HK and FK isoforms in an antisense (AS301), a control (Ctrl3) and a sense (S111) clones, using anion-exchange chromatography.

Analyses were carried out on protein extracts from clones AS301 (a,d), Ctrl3 (b,e) and S111 (c,f). After desalting on PD-10 columns, proteins (2 mg) were loaded on a 6-mL DEAE Fractogel column, and eluted using a KCl gradient (*straight line*). The elution profiles (*filled circles*) of GK (a–c) and FK (d–f) activities are plotted as functions of the fraction number (1 mL per fraction). GK (a–c) and FK (d–f) activities (in mU fraction^{-1}) were measured with 5 mM Glc and 5 mM Fru, respectively. HK activity (a–f) was resolved as two peaks 1 and 2, whereas FK activity (d–f) eluted as peak 3.

4.6.3 Enzyme activities of primary metabolism

The impact of HK activity alteration on 18 enzyme activities of primary metabolism was surveyed in two antisense (AS213, AS301), two control (Ctrl2, Ctrl3) and two sense (S101, S111) clones (Table 4.1). Activity values obtained for Ctrl2 and Ctrl3 were generally comparable to those obtained by others in roots, germinating seeds or suspension cells. This held true for NAD:GAPDH (Journet *et al.*, 1986), ALD and FBPase (Moorhead *et al.*, 1994), NADP:GAPDH, PFK, PGI and UGPase (Borchert *et al.*, 1993), HK and PFP (Bouny and Saglio, 1996), PK and PEPase (Carswell *et al.*, 1996), SuSy (Kim *et al.*, 2000), AGPase and PGM (Fernie *et al.*, 2001), PEPC (Tesfaye *et al.*, 2001), TPI (Dorion *et al.*, 2005), and INVs (Sung *et al.*, 1989; Tang *et al.*, 1999; Kim *et al.*, 2000). In addition, total INV activity was much higher than SuSy activity in the clone population (Table 4.1), which corroborated observations made on maize roots (Kim *et al.*, 2000). Only the HK activity varied significantly among the six clones, no consistent changes appeared among the other enzyme activities. Therefore, the clone population was suitable for metabolic control analysis because HK activity was modified without perturbation of other primary metabolism enzymes (Fell, 1992; Ap Rees and Hill, 1994).

Table 4.1: Specific activities of primary metabolism enzymes assayed in two antisense (AS213, AS301), two control (Ctrl2, Ctrl3) and two sense (S101, S111) clones.

Enzyme	Specific activities (mU mg protein^{-1}) ± SE					
	AS213	AS301	Ctrl2	Ctrl3	S101	S111
AGPase	20 ± 6	17 ± 6	30 ± 7	15 ± 1	16 ± 2	20 ± 4
UGPase	918 ± 173	896 ±177	1042 ± 63	698 ± 38	826 ± 73	1033 ± 67
SuSy	238 ± 20	228 ± 30	244 ± 10	212 ± 20	210 ± 20	263 ± 30
acid INV	28 ± 2	14 ± 2	19 ± 1	21 ± 2	26 ± 3	10 ± 1
neutral INV	2 ± 1	2 ± 1	1 ± 1	4 ± 1	4 ± 1	2 ± 1
cell wall INV	735 ± 50	461 ± 2	358 ± 30	714 ± 80	754 ± 40	321 ± 30
GK	**14 ± 1**	**15 ± 1**	**19 ± 2**	**19 ± 2**	**89 ± 8**	**95 ± 8**
FK	**22 ± 1**	**21 ± 1**	**20 ± 1**	**24 ± 2**	**89 ± 6**	**101 ± 4**
PGI	652 ± 30	621 ± 80	808 ± 70	596 ± 0	641 ± 30	728 ± 30
PGM	256 ± 10	258 ± 20	274 ± 20	245 ± 10	242 ± 10	273 ± 20
PFK	65 ± 4	64 ± 4	64 ± 4	65 ± 3	64 ± 2	85 ± 4
FBPase	15 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	16 ± 1
PFP	262 ± 10	252 ± 10	286 ± 20	262 ± 10	253 ± 10	270 ± 10
ALD	47 ± 0	49 ± 10	43 ± 10	46 ± 0	47 ± 0	46 ± 0
TPI	1490 ± 90	1810 ± 40	1850 ± 160	1660 ±140	1800 ±110	1480 ± 60
NAD:GAPDH	1450 ± 140	1400 ±160	1530 ± 80	1480 ±110	1540 ± 60	1550 ± 40
NADP:GAPDH	3 ± 1	2 ± 1	3 ± 1	3 ± 1	2 ± 0	3 ± 1
PK	412 ± 50	338 ± 20	363 ± 20	370 ± 30	412 ± 20	428 ± 30
PEPC	112 ± 3	72 ± 2	100 ± 8	105 ± 4	114 ± 4	95 ± 4
PEPase	142 ± 5	144 ± 7	137 ± 11	144 ± 8	147 ± 4	167 ± 8

Data are means ± SE of quadruplicates from four to five independent experiments. Only HK specific activity was modified, and is represented in boldface. AGPase, ADP-Glc pyrophosphorylase; UGPase, UDP-Glc pyrophosphorylase; SuSy, D-sucrose synthase; INV, invertase; GK, glucokinase; FK, fructokinase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; PFK, ATP-dependent phosphofructokinase; FBPase, D-fructose-1,6-bisphosphatase; PFP, pyrophosphate-D-fructose-6-phosphate 1-phosphotransferase; TPI, triosephosphate isomerase; NAD(P):GAPDH, NAD(P)$^+$-dependent D-glyceraldehyde-3-phosphate dehydrogenase; PK, pyruvate kinase, PEPC, phospho*enol*pyruvate carboxylase; PEPase, phospho*enol*pyruvate phosphatase.

4.6.4 Alteration of HK activity markedly affects root growth

We observed a pronounced growth phenotype among the transgenic root clones (Fig. 4.3a). This prompted us to quantify the root total length, tip number and mean diameter for most of the clone population after 14 days of growth (Fig. 4.3b,c,d). The root total length and tip number tended to decrease with rising HK activities, leading to significant differences between mean values from sense and antisense clones (Fig. 4.3b,c). Antisense clones exhibited larger changes in growth behavior than sense clones despite lesser variations from control values of HK activity (Fig. 4.3a,b). This may point to a threshold of HK activity below which root elongation may be greatly enhanced, whereas HK activity increases above that threshold are less efficient in inhibiting root growth. Average root diameter remained constant in the clone population (Fig. 4.3d). So did the total length/tip number ratio, suggesting that the branching pattern of the clones was not influenced by HK (data not shown). Importantly, there was no evidence of necrosis or senescence in the slowly-growing sense clones, which continued to grow at a steady pace for 10 days after the experiment and could be successfully subcultured. Therefore, our study provides the first evidence for a role of root HK in growth inhibition, but not in triggering senescence, in this heterotrophic organ. Root growth inhibition by elevated HK activity has been observed in tomato, but was clearly attributed to HK expression and activity in photosynthetic tissues (Dai *et al.*, 1999). Such possibility can be totally ruled out here since the root clones were devoid of any aerial part. Other transgenic approaches have revealed contrasting differences in the importance of HK in heterotrophic metabolism. Thus, the yield and carbohydrate metabolism of potato tuber remained unaffected despite a 7- to 8-fold variation in HK activity levels (Veramendi *et al.*, 1999; Veramendi *et al.*, 2002). On the contrary, increased HK activity reduced tomato fruit and seed size, possibly due to low respiratory rates and ATP/ADP ratios (Menu *et al.*, 2004). Therefore, heterotrophic organs need to be studied as separate systems in order to establish whether or not HK is of importance in governing their primary metabolism and growth. We hypothesized that the manipulation of HK activity in the root clones may have perturbed their carbohydrate metabolism, thus affecting their growth. This led us to further investigate the metabolite content and the glycolytic flux at the step of Glc phosphorylation in a subgroup of root clones.

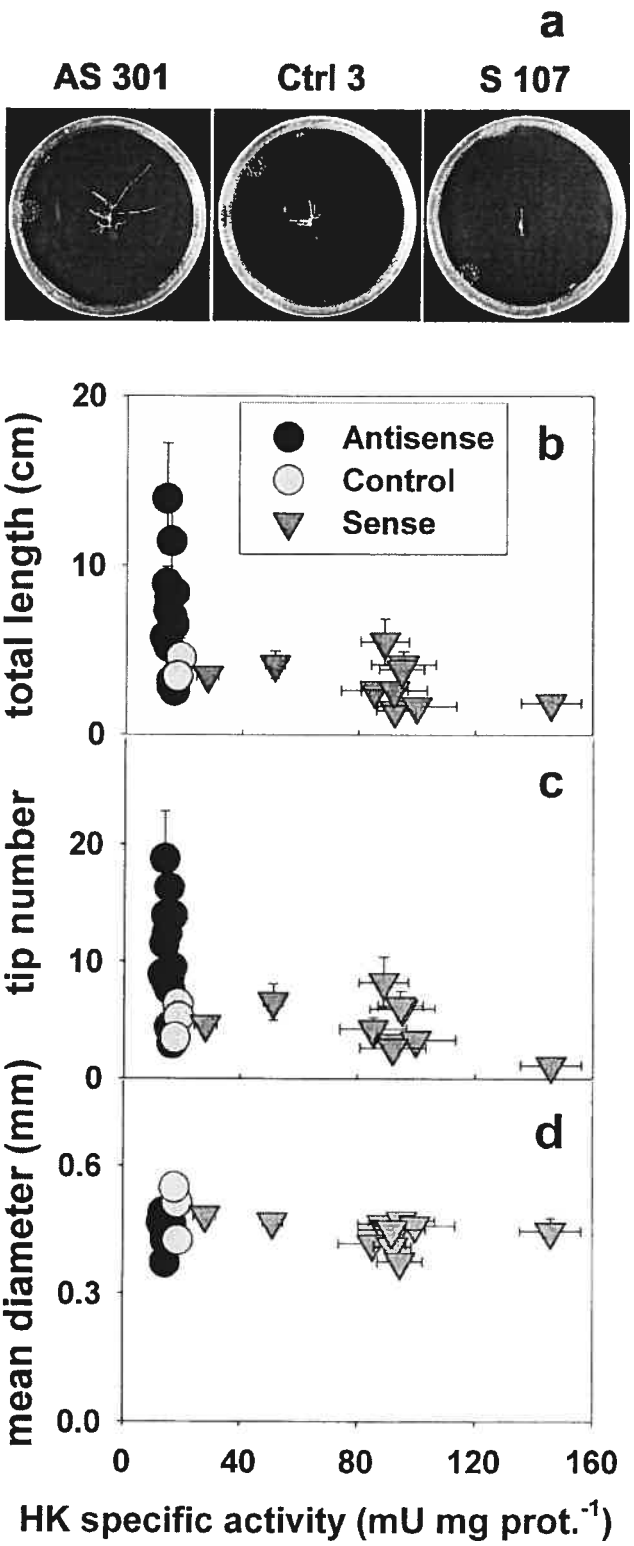

a
AS 301
Ctrl 3
S 107
Antisense
Control
Sense
b
c
d
total length (cm)
tip number
mean diameter (mm)
20
10
0
20
10
0
0.6
0.3
0.0
0
40
80
120
160
HK specific activity (mU mg prot.-1)

Figure 4.3: Effects of altered HK activity levels on potato root growth.

a, Photographs of an antisense (AS301), a control (Ctrl3) and a sense (S107) clones grown. for 14 days. All root tips were 0.6 cm long on day 0. b–d, Altering HK activity levels in potato roots effected their total root length and tip number, but not their root diameter. Total root length (b), tip number (c) and mean diameter (d) were assessed for thirteen antisense (*black circles*), three control (*grey circles*) and ten sense (*grey triangles*) clones after 14 days of growth on solid MS medium. Growth conditions and measurements are described in Experimental procedures. Y values are means ± SE of quadruplicates from two independent experiments, X values are from Fig. 4.1. Mean values of total root length and tip number were significantly different ($P < 0.02$) between sense and antisense clones, based on Wilcoxon's rank sum test.

4.6.5 Root metabolite contents

The influence of HK on root metabolism was examined by quantifying the pool sizes of sugars (Fig. 4.4), organic acids (Fig. 4.5) and amino acids (Fig. 4.6) in twelve antisense, three control and nine sense clones. Levels of hexose-Ps were quantified using spectrophotometric assays in two representative antisense, control and sense clones (Table 4.2). Glc, Fru and Suc levels in the control clones (Fig. 4.4) were in the same range as those found in carrot (*Daucus carota*) and maize roots (Tang and Sturm, 1999; Kim *et al.*, 2000). Glc and Fru levels tended to decrease with increasing HK activities, and significant differences were observed between mean values obtained from sense and antisense clones (Fig. 4.4a,b). This suggests that both Glc and Fru served as substrates for HK in the root clones, despite the much lower affinity of plant HK for Fru than for Glc (Claeyssen and Rivoal, 2007). Suc levels remained unchanged among the transgenics (Fig. 4.4c), thus contrasting with the decline in Glc and Fru pools. Furthermore, Glc levels were twice as large as those of Fru throughout the range of HK activity values (Fig. 4.4d). These data, therefore, agree with changes in HK activity levels in the clones occurring without changes in INV and SuSy activities for commensurate hexose supply to the HK reaction (Table 4.1). Hexose-Ps were assayed in two antisense (AS213, AS301), two control (Ctrl2, Ctrl3) and two sense (S101, S111) clones (Table 4.2). Levels of D-fructose-6-P (F6P), G1P and G6P were remarkably similar between the different clones considering their large differences in HK activities.

With respect to organic acids, shikimate was investigated as well as the tricarboxylic acid (TCA) cycle components malate, isocitrate and fumarate (Fig. 4.5). Malate levels in most antisense clones were significantly higher than in control and sense clones (Fig. 4.5a), suggesting its increased synthesis and/or decreased utilization in the HK underexpressors. Increased pool sizes of malate in antisense clones may point to carbon supply and energy yield being sufficient for use of malate as a storage compound of carbon and reducing equivalents. Its subsequent metabolization, e.g. via malate dehydrogenase (EC 1.1.1.37) or NAD-malic enzyme (EC 1.1.1.38), could then serve for NADH production. (Dieuaide-Noubhani *et al.*, 1995; Scheibe, 2004). Levels of isocitrate and fumarate were unaltered among the clone population (Fig. 4.5b,c), as were those of shikimate (Fig. 4.5d).

The latter is the precursor of the aromatic amino acids Phe, Trp and Tyr (Herrmann and Weaver, 1999). Its carbon backbone is derived from phospho*enol*pyruvate (PEP) and D-erythrose-4-phosphate, which are intermediates in glycolysis and the oxidative pentose-phosphate pathway (OPPP), respectively (Herrmann and Weaver, 1999). Therefore, unaltered levels of shikimate in the clone population were not suggestive of a change in carbon supply from glycolysis or the OPPP.

Table 4.2: Hexose-phosphate levels in two antisense (AS213, AS301), two control (Ctrl2, Ctrl3) and two sense (S101, S111) clones.

Data are means ± SE of five to eighteen separate extractions. F6P, D-fructose-6-phosphate; G1P, D-glucose-1-phosphate; G6P, D-glucose-6-phosphate.

Metabolite	Tissue content (nmol. g^{-1} FW) ± SE					
	AS213	AS301	Ctrl2	Ctrl3	S101	S111
G6P	490 ± 155	387 ± 111	447 ± 111	447 ± 115	438 ± 103	512 ± 128
F6P	223 ± 70	195 ± 61	232 ± 64	237 ± 65	231 ± 54	260 ± 67
G1P	192 ± 86	195 ± 56	217 ± 54	214 ± 59	193 ± 49	243 ± 62

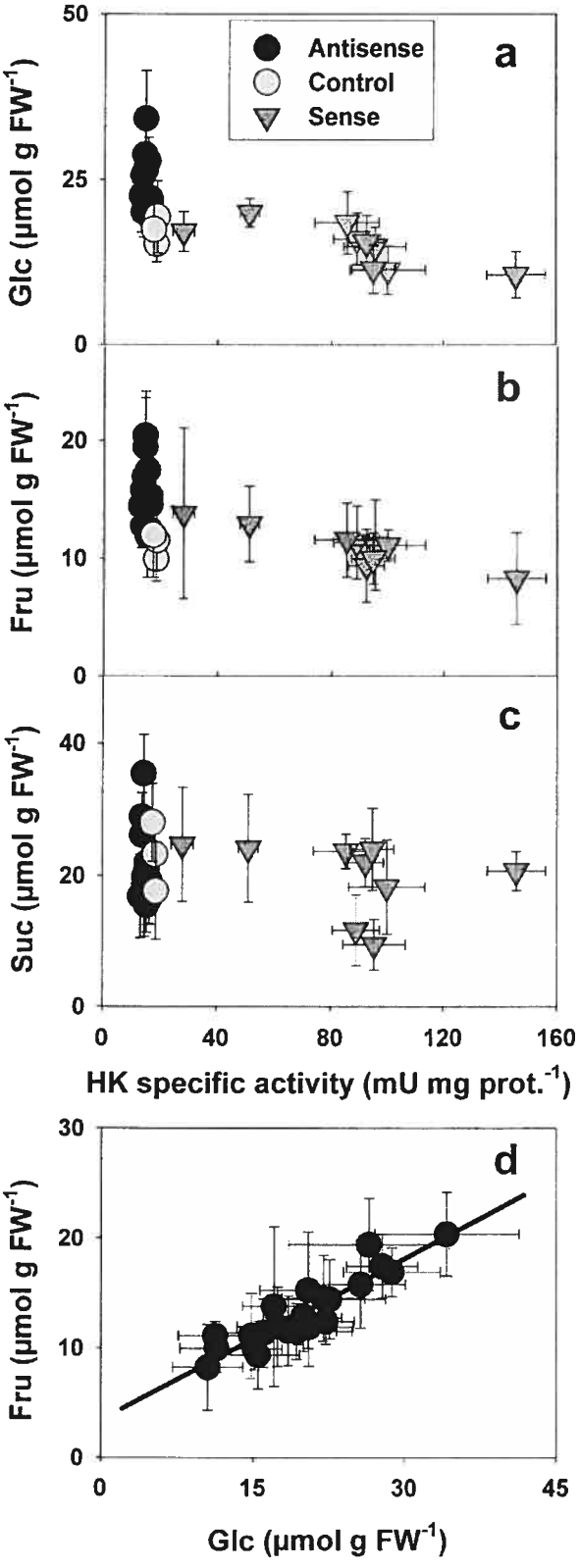

Antisense
Control
Sense
a
b
c
d
Glc (µmol g FW^{-1})
Fru (µmol g FW^{-1})
Suc (µmol g FW^{-1})
HK specific activity (mU mg prot.$^{-1}$)
Fru (µmol g FW^{-1})
Glc (µmol g FW^{-1})

Figure 4.4: Sugar contents in potato roots with altered HK specific activities.

Levels of Glc (a), Fru (b) and Suc (c) were determined for twelve antisense (*black circles*), three control (*grey circles*) and nine sense (*grey triangles*) clones. Y values are means ± SE from five to eight separate experiments, X values are from Fig. 4.1. Mean values of Glc and Fru contents were significantly different ($P < 0.02$) between sense and antisense clones, based on Wilcoxon's rank sum test. d, Glc and Fru levels were correlated among root clones, following a linear relation: [Fru] = 0.49 [Glc] + 3.5 ($R^2 = 0.83$).

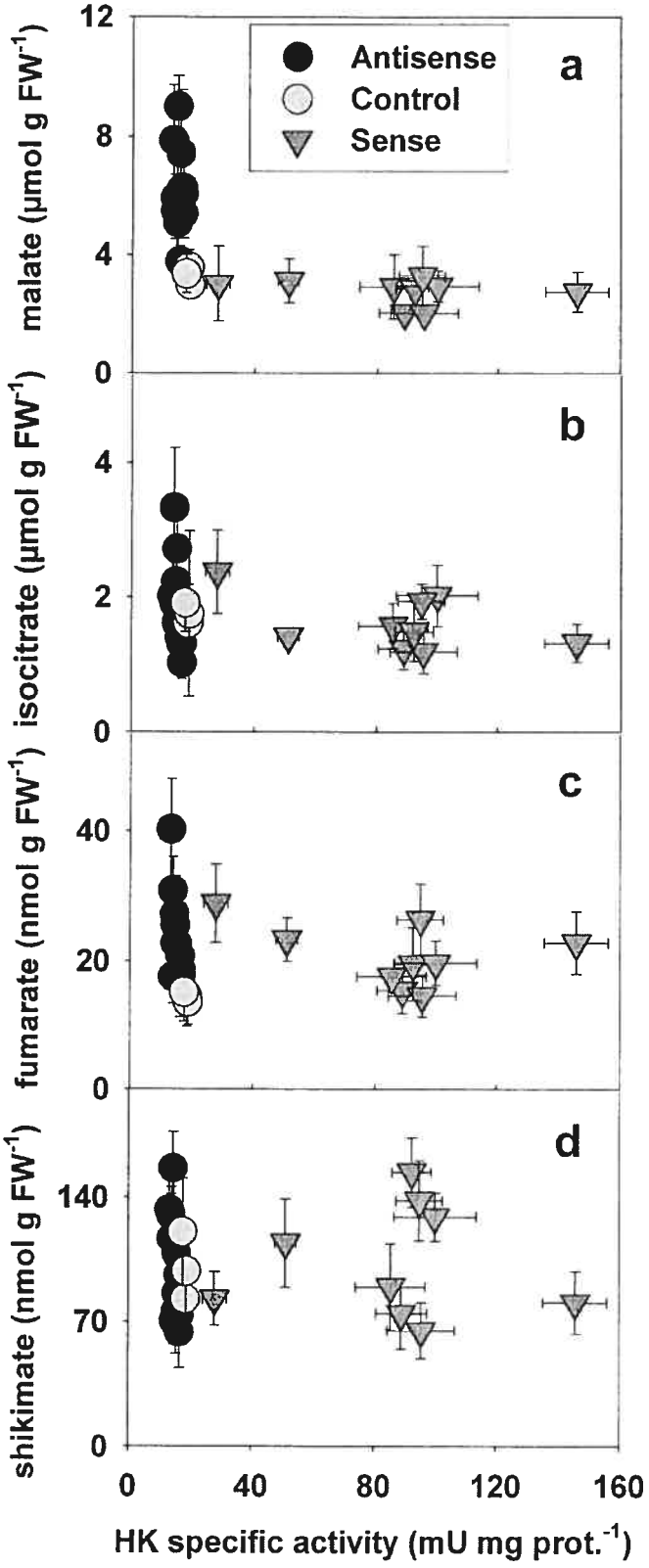
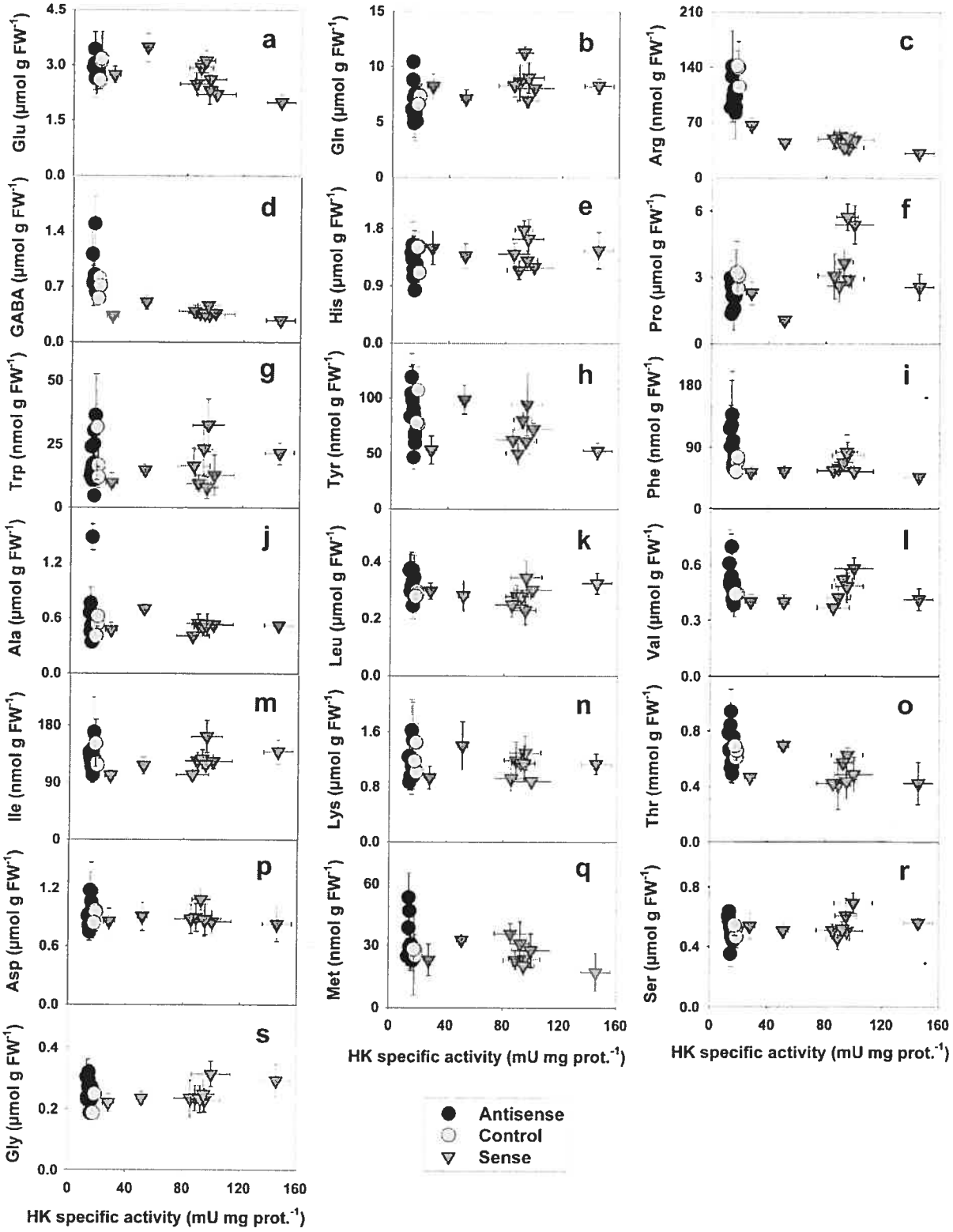

Antisense
Control
Sense
a
b
c
d
malate (µmol g FW-1)
isocitrate (µmol g FW-1)
fumarate (nmol g FW-1)
shikimate (nmol g FW-1)
HK specific activity (mU mg prot.-1)
0
4
8
12
2
40
20
140
70
80
120
160

Figure 4.5: Organic acid contents in potato roots with altered HK specific activities.

Levels of malate (a), isocitrate (b), fumarate (c) and shikimate (d) were quantified for twelve antisense (*black circles*), three control (*grey circles*) and nine sense (*grey triangles*) clones. Y values are means ± SE from five to eight separate experiments, X values are from Fig. 4.1. Mean values of malate contents were significantly different ($P < 0.02$) between sense and antisense clones, according to Wilcoxon's rank sum test.

Nineteen amino acids were surveyed in the root clones (Fig. 4.6). Total amounts of nitrogen in these amino acids were stable across the entire transgenic population (31.5 ± 0.7 μmol N g FW^{-1}, not shown), indicating that HK manipulation did not impact on global nitrogen assimilation. Glu levels were unaltered whereas there was a slight increase in Gln with rising HK activities, leading to significant differences between mean values from sense and antisense clones (Fig. 4.6a,b). Glu and Gln are important intermediates of the Gln synthetase/Glu synthase (GS/GOGAT) cycle that drives the assimilation of nitrogen in roots (Lea and Miflin, 2003). Cytosolic GS1 (EC 6.3.1.2) fixes ammonium onto Glu to yield Gln in an ATP-consuming reaction. Then, Gln and the TCA cycle intermediate α-ketoglutarate (α-KG) are transformed into two Glu molecules by plastidic GOGAT (EC 1.4.1.13), in an NADH-dependent manner (Lea and Miflin, 2003). The GS/GOGAT cycle thus incorporates nitrogen which is then distributed into the Glu-derived amino acids Arg, His, Pro and γ-aminobutyric acid (GABA). There was a marked trend towards a decrease in Arg and GABA levels when HK activities increased, with significant differences between mean values from sense and antisense clones (Fig. 4.6c,d). His and Pro contents remained unaffected among the clone population (Fig. 4.6e,f). It may be proposed that increasing HK activities resulted in enhanced utilization or degradation of Arg and GABA, although without beneficial effects on growth (Figs. 4.3 and 4.6c,d). More plausibly, these metabolites are known nitrogen stores in plants (Shelp *et al.*, 1999; Slocum, 2005). Therefore, their levels may readily decline unless growth conditions are optimal. Carbon flow from α-KG to succinate is less energy efficient through the GABA shunt for GABA synthesis than through the TCA cycle (Shelp *et al.*, 1999). Similarly, the Arg biosynthetic pathway consumes ATP and NADPH (Slocum, 2005). Should increased HK activities lead to energy shortage, it would be conceivable that the TCA cycle be dedicated to NADH and ATP production, at the expense of energy-costly biosyntheses of the nitrogen stores Arg and GABA. A reduced carbon flow from α-KG through the GS/GOGAT cycle to Arg and GABA biosyntheses would explain the tendency for Gln to accumulate concomitantly (Fig. 4.6b,c,d). Alternatively, GABA is known to be actively synthesized under various biotic and abiotic stress conditions in plants (Shelp *et al.*, 1999; Bouché *et al.*, 2004). The altered GABA contents in the transgenic roots may indicate some interference of HK manipulation with elements of stress signaling pathways (Fig. 4.6d). With respect to that possibility,

investigation of reactive O_2 species and superoxide ion levels (Bouché *et al.*, 2004) may reveal useful information regarding the potato root clones. GABA synthesis is highly dependent on Glu decarboxylase, a Ca^{2+}/calmodulin-regulated enzyme (Bouché *et al.*, 2004). A testable hypothesis is that observed differences in GABA contents could be due to differences in Ca^{2+} metabolism in the transgenic population (Fig. 4.6d). With respect to aromatic amino acids, the levels of Trp and Tyr were constant among the root clones (Fig. 4.6g,h). However, average Phe contents in sense clones were significantly lower than those in antisense clones (Fig. 4.6i). As mentioned previously, the levels of their precursor shikimate were unaltered in the transgenics (Fig. 4.5d). However, a shortage of other precursors situated downstream, namely PEP, Glu, or ATP, may have hampered Phe synthesis (Herrmann and Weaver, 1999). The levels of Ala, Leu and Val (pyruvate family, Fig. 4.6j–l), of Ile, Lys, Asp and Met (derived from oxaloacetate, OAA, Fig. 4.6m,n,p,q), and of Ser and Gly (derived from D-3-phosphoglycerate, Fig. 4.6r,s), were constant among the transgenics. As for OAA-derived Thr, the average levels for HK-overexpressing clones were significantly lower than those for HK-underexpressing clones (Fig. 4.6o). These results may again point to shortage of OAA, NADH or ATP needed for the synthesis of this amino acid (Azevedo *et al.*, 1997). Collectively, the data may suggest an effect of altered HK activities on the supply of ATP, NADH, or the TCA cycle components OAA and α-KG, for the synthesis of Arg, GABA, Phe and Thr.


a
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Glu (µmol g FW-1)
Gln (µmol g FW-1)
Arg (nmol g FW-1)
GABA (µmol g FW-1)
His (µmol g FW-1)
Pro (µmol g FW-1)
Trp (nmol g FW-1)
Tyr (nmol g FW-1)
Phe (nmol g FW-1)
Ala (µmol g FW-1)
Leu (µmol g FW-1)
Val (µmol g FW-1)
Ile (nmol g FW-1)
Lys (µmol g FW-1)
Thr (mmol g FW-1)
Asp (µmol g FW-1)
Met (nmol g FW-1)
Ser (µmol g FW-1)
Gly (µmol g FW-1)
HK specific activity (mU mg prot.-1)
HK specific activity (mU mg prot.-1)
Antisense
Control
Sense

Figure 4.6: Amino acid contents in potato roots with altered HK specific activities.

Levels of Glu (a), Gln (b), Arg (c), γ-aminobutyric acid (GABA) (d), His (e), Pro (f), Trp (g), Tyr (h), Phe (i), Ala (j), Leu (k), Val (l), Ile (m), Lys (n), Thr (o), Asp (p), Met (q), Ser (r) and Gly (s) were determined in twelve antisense (*black circles*), three control (*grey circles*) and nine sense (*grey triangles*) clones. Y values are means ± SE from five to eight separate experiments, X values are from Fig. 4.1. Mean values of Gln, Arg, GABA, Thr and Phe contents were significantly different ($P < 0.02$) between sense and antisense clones, according to Wilcoxon's rank sum test.

4.6.6 HK exerts tight control over the Glc-to-G6P flux

The glycolytic flux at the step of Glc phosphorylation was measured in five antisense, three control and eight sense clones, using the labeled Glc analog [U-^{14}C]DOG as a tracer. DOG is taken up by plant cells and phosphorylated by HK into DOG6P. Experimental evidence shows that further metabolization of [^{14}C]DOG is extremely slow (Klein and Stitt, 1998). Accordingly, [U-^{14}C]DOG6P levels rose linearly between 0 and 6 h of labeling with [U-^{14}C]DOG, and declined thereafter (data not shown). Therefore, the Glc-to-G6P flux was quantified from the rates of [U-^{14}C]DOG6P accumulation and the specific radioactivity of Glc in samples incubated with [U-^{14}C]DOG for 2 h. Catabolism of [U-^{14}C]DOG6P was minimal within this time frame (data not shown, see also Klein and Stitt, 1998) and hence, underestimation of the glycolytic flux was unlikely. It has been shown in animals that the affinity of Glc transporters and HK may differ between DOG and Glc (Grossbard and Schimke, 1966). Such differences are corrected for by *in vivo* measurement of the lumped constant for DOG (Utriainen *et al.*, 2000). However, we are unaware of any measurement in plants of the lumped constant for DOG or the K_m(DOG) of HK. Therefore, we assumed that the lumped constant for DOG was 1 in our experiments, i.e. that the various HK isoforms of potato roots had similar affinities to Glc and DOG.

The natural logarithm of the Glc-to-G6P flux was plotted against the natural logarithm of HK activity to assess the FCC of HK over Glc phosphorylation (Fig. 4.7). A linear regression of the data was used for calculation of the FCC from the slope as the relationship between ln(flux) and ln(HK activity) was assumed to be linear over the range of HK activities (Fell, 1992). The FCC of HK over Glc phosphorylation was found at the remarkably high value of 0.76 ± 0.08 (Fig. 4.7). To our knowledge, this is the first report on the level of control of HK over the plant glycolytic pathway. Furthermore, a high (0.7 to 1) FCC of HK over glycolysis or its upper segment has been reported in various mammalian tissues and cell types (Rapoport *et al.*, 1974; Meléndez-Hevia *et al.*, 1992; Kashiwaya *et al.*, 1994; Sweet and Matschinsky, 1995; Puigjaner *et al.*, 1997; Wang and Iynedjian, 1997). Our results now provide evidence for similar FCCs of HK both in plants and mammals, despite major differences in the structure and regulation of their glycolytic pathways (Plaxton, 1996). In yeast, a lower (0.2 to 0.5) FCC has been assigned to HK,

which may be due to its inhibition by T6P (Aon and Cortassa, 1998; Ernandes *et al.*, 1998). It has been proposed that glycolytic regulation by T6P has evolved specifically to cope with sudden and large variations in Glc availability, in contrast to mammals where Glc levels are relatively constant (Teusink *et al.*, 1998). In support of that view, a model involving direct product inhibition (by G6P) of HK as in mammals did not perform as well as the T6P system at high and low Glc levels (Teusink *et al.*, 1998). In plants, only some HK isoforms have been found sensitive to G6P inhibition and none to T6P (Claeyssen and Rivoal, 2007). Therefore, our data and those from yeast and animal studies suggest that in all Eukaryotes, HK is a major control point at the gate of the glycolytic pathway. However, HK may be involved in glycolytic regulation in a unique manner in plants, which is further discussed below.

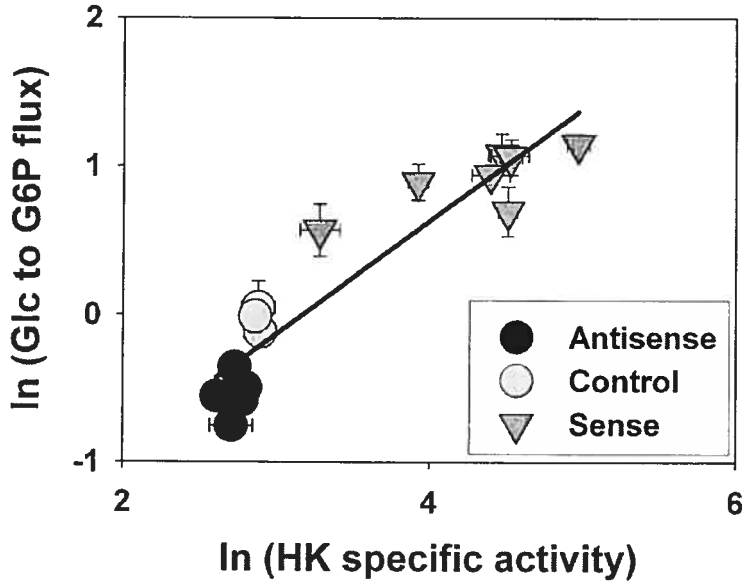

Figure 4.7: Determination of the flux control coefficient of HK over the phosphorylation of Glc in potato roots.

Glycolytic flux from Glc to D-glucose-6-phosphate (G6P) was quantified in five antisense (*black circles*), three control (*grey circles*) and eight sense (*grey triangles*) clones. Y values are means ± SE of triplicates from three to five separate experiments, X values are natural logarithm values of HK activities from Fig. 4.1.

4.6.7 Evidence that G6P metabolism was altered as a result of HK manipulation

We examined the extent to which G6P was metabolized in the five antisense, three control and eight sense clones used for flux and FCC measurement (Fig. 4.8). G6P contents were deduced from the specific radioactivity of Glc and the [U-^{14}C]DOG6P levels found in samples labeled with the tracer for 2 or 8 h. As mentioned above, [U-^{14}C]DOG6P levels rose steadily between 0 and 6 h of labeling, and declined thereafter (data not shown). Interestingly, [U-^{14}C]DOG6P exhibited a similar time-course evolution in *Chenopodium rubrum* cell suspensions labeled with [U-^{14}C]DOG (Klein and Stitt, 1998). In these cells, the decline of [U-^{14}C]DOG6P after 6 h of labeling could not be due to its metabolization into glycolysis as it lacks a hydroxyl group at the C-2 position. Instead, a large proportion of it was shown to be recycled to [U-^{14}C]DOG, to 2-deoxy-D-[U-^{14}C]sucrose (30–35%) and, later on, to unidentified compounds (Klein and Stitt, 1998). These data suggest that Glc/Glc-P and Suc cycles took place in the *Chenopodium* cells, and could operate with DOG and DOG6P. *Chenopodium* cells may thus contain Glc-P phosphatase activities and Suc-synthesizing activities that use Glc-P and non-physiological DOG6P as substrates. In our potato root clones, G6P was expected to behave similarly to its analog DOG6P. Therefore, the decline in G6P levels matching that in [U-^{14}C]DOG6P levels between 2 and 8 h of labeling likely reflected the metabolization of G6P via Glc/Glc-P and/or Suc cycling. Our labeling experiments, originally designed for flux measurement at steady state, only allowed for rough estimates of G6P amounts in samples labeled for 8 h as those were no longer at isotopic steady state. These estimates were nonetheless meaningful as G6P losses between 2 and 8 h of labeling appeared proportional to HK activity levels in the root clones (Fig. 4.8). Thus, antisense clones displayed G6P losses as low as 0.5 µmol G6P g FW^{-1} in 6 h whereas the S104 clone, with an HK activity level 8 times higher than in control clones, metabolized over 4 µmol G6P g FW^{-1} in 6 h. This is a likely possibility since G6P-to-Glc and G6P-to-Suc fluxes added up to 2.7 µmol G6P min^{-1} g FW^{-1} in maize roots, thereby metabolizing over 900 µmol G6P g FW^{-1} in 6 h (Alonso *et al.*, 2005). Taken together, our data suggest that G6P was metabolized via Glc/Glc-P and/or Suc cycling in the potato root clones, to an extent that was proportionate with the Glc-to-G6P flux.

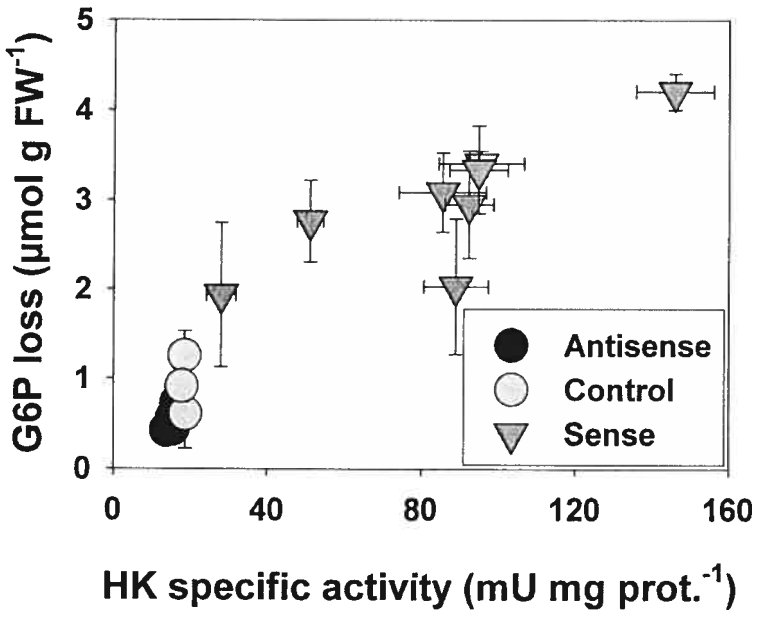

Figure 4.8: Effect of altered HK activity on metabolization of D-glucose-6-phosphate in potato roots.

D-glucose-6-phosphate (G6P) levels were estimated in five antisense (*black circles*), three control (*grey circles*) and eight sense (*grey triangles*) clones after 2 and 8 h of labeling with 2-deoxy-D-[U-^{14}C]glucose ([U-^{14}C]DOG). Samples were at metabolic and isotopic steady state between 0 and 6 h of labeling, as evidenced by linear accumulation of HK-yielded [U-^{14}C]DOG6P over time. Therefore, G6P levels deduced from those of [U-^{14}C]DOG6P provided a good estimate of G6P amounts produced by HK in samples labeled for 2 h. In those labeled for 8 h, which were not at steady state, G6P levels were reduced presumably due to G6P metabolization. The estimated loss in G6P between 2 and 8 h of labeling is represented for the various clones studied.

4.6.8 Proposed mechanism of regulation by HK of plant root glycolysis

The changes in growth behavior and metabolite contents (Figs. 4.3–4.7) have raised the question of whether the catalytic and/or sensing function of HK was affected in our transgenics. Previous manipulations of either function by transformation have led to dramatic perturbations in growth and metabolism of several plant systems (Jang *et al.*, 1997; Dai *et al.*, 1999; Veramendi *et al.*, 1999; Veramendi *et al.*, 2002; Moore *et al.*, 2003; Menu *et al.*, 2004). However, it has most often been difficult to discriminate between effects due to impaired sugar signaling and deregulated metabolism downstream of HK, unless both functions were adequately uncoupled (Halford *et al.*, 1999; Moore *et al.*, 2003). The levels of hexoses, but not Suc, varied among the root clones (Fig. 4.4), which may have impacted on the multiple sugar signaling pathways and contributed to the growth phenotype (Freixes *et al.*, 2002; Rolland *et al.*, 2006). It may also be argued that sense and antisense clones exhibited contrasting HK isoform profiles (Fig. 4.2) and hence, may have been differently affected in the catalytic and sensing functions of HK. Nevertheless, a linear relationship was described between HK activity levels and parameters of root growth (length, number of tips) with a moderate but significant Pearson correlation coefficient of -0.54. The correlation between HK activity and summed levels of Glc, Fru, malate, Arg, GABA, Thr, and Phe was even higher, with a Pearson coefficient of -0.73 (data from Figs. 4.4–4.6). This contrasted with the null correlation between HK activity and quantitative Glc responses observed in *Arabidopsis* mutants deficient in the sugar sensing function of HK (Moore *et al.*, 2003). Therefore, our results strongly suggest that the changes in growth behavior and in metabolite pools were due mostly to the manipulation of the catalytic function of HK, regardless of the HK isoform profiles of the root clones.

It appears from our data that HK is a major control step at the entry of hexoses to glycolysis (Fig. 4.7), and raising HK activity among the root clones did stimulate Glc and Fru influx into the glycolytic pathway (Fig. 4.4a,b). Surprisingly, however, this was accompanied by unchanged hexose-P levels (Table 4.2), as well as limited growth and decreased levels of several amino acids (Figs. 4.3 and 4.6), suggesting a possible deregulation of glycolysis rather than a rise in its flux. There was also evidence to suggest the operation in our transgenic roots of the Suc and/or Glc/Glc-P cycles, which involve HK

(Nguyen-Quoc and Foyer, 2001; Alonso *et al.*, 2005). Indeed, it appeared from data on DOG6P catabolism that G6P was not only catabolized through glycolysis but also recycled back to Glc and/or Suc in the root clones (Fig. 4.8). The disappearance of G6P was greater in sense than in control or antisense clones. A likely explanation for this situation is that sense clones recycled Glc-P to Glc and/or Suc at higher rates. Sustained Suc cycling may be envisaged in our sense clones as HK has been shown to exert exclusive control over Suc cycles with an FCC near 1 in sugar cane culm (Rohwer and Botha, 2001). Furthermore, Suc degradation provided the HK reaction with equal amounts of Glc and Fru via INVs, and only with Fru via SuSy. Since the only altered activity in the root clones was that of HK (Table 4.1), it was expected to impact mainly on Glc pools due to its higher affinity for Glc compared to Fru (Claeyssen and Rivoal, 2007). Nonetheless, both Glc and Fru contents decreased with rising HK activities, and Glc levels remained twice as large as those of Fru in all clones (Fig. 4.4a,b,d). The data may be reconciled by taking in account the increased demand in Glc-P for Glc and especially Suc re-syntheses, without commensurate increases in Suc degradation activities (Table 4.1). The Fru initially phosphorylated by HK or FK (EC 2.7.1.4) could thus feed the Glc pool via Glc/Glc-P and Suc cycles. These cycles have been proposed to stimulate glycolytic flux by raising ATP demand, given their capacity to consume great amounts of ATP in plant cells (Fernie *et al.*, 2002; Urbanczyk-Wochniak *et al.*, 2003; Alonso *et al.*, 2005). Accordingly, ATP demand seems to be involved in control of glycolytic flux in bacteria, yeast, animals and plants (Thomas and Fell, 1998; Larsson *et al.*, 2000; Koebmann *et al.*, 2002; Sweetlove *et al.*, 2002; Liu *et al.*, 2006). From our survey of growth behavior and of a large number of metabolite pools, however, we do not have evidence for increased glycolytic flux in potato roots despite indications of increased Glc/Glc-P and Suc cycling with increasing HK activities. Therefore, we agree with Junker *et al.* (2006) that if Suc cycling participates in raising glycolytic flux, then additional, unidentified factors must be involved. Glc/Glc-P and Suc cycles may rather introduce a layer of glycolytic regulation via the sensitivities of HK, Glc-P phosphatase and Suc-metabolizing enzymes to specific effectors, e.g. ADP or G6P for HK (Claeyssen and Rivoal, 2007). These cycles may constitute a built-in regulator that integrates hexose availability and downstream metabolic activity via effector levels, to adjust hexose-P levels

for adequate glycolytic activity. Future investigation of the enzymatic and molecular aspects of such a regulatory mechanism will undoubtedly clarify its function.

At this stage, it is of interest to examine how this regulatory system may have been challenged in the transgenic roots. In the antisense clones, reduced HK activity may have lessened ATP dissipation by Glc/Glc-P and Suc cycling, while still injecting sufficient hexose into glycolysis for sustaining pools of downstream metabolites (Figs. 4.5 and 4.6). In absence of incoherence in the overall metabolism, the spared ATP could then be allocated to biosyntheses, thereby improving growth (Figs 4.3 and 4.6). In the sense clones, higher amounts of HK activity seemed to trigger commensurate increases in Glc-P metabolism via Glc/Glc-P and Suc cycling (Fig. 4.8). These processes may thus be involved in glycolytic regulation by maintaining a stable pool of hexose-Ps, even in the presence of high amounts of functional HK. This may be particularly important for two reasons. Firstly, the inherent risk of Glc-accelerated death due to the autocatalytic principle of the glycolytic pathway may not only apply to yeast, but also to other Eukaryotes (Teusink *et al.*, 1998; Iynedjian, 1998). Accordingly, potent inhibition of mammalian HKs I–III by G6P has been proposed to avoid the risk of P_i sequestration in hexose-Ps and subsequent Glc-accelerated death in most mammalian cell types (Iynedjian, 1998; Wilson, 2003). In hepatocytes, the predominant HK is HK IV ('glucokinase'), which is not product inhibited by G6P. The 'guard at the gate' of hepatic glycolysis may then be provided by Glc/Glc-P cycling and by HK IV inhibition by a glucokinase-regulatory protein (GKRP) (Iynedjian, 1998). ScHK2 used in this study was shown to be insensitive to G6P inhibition (Claeyssen *et al.*, 2006), a feature shared with the majority of plant HKs characterized thus far (Claeyssen and Rivoal, 2007). The action of the Glc/Glc-P and Suc cycles may then be seen as a way to dampen the effects of varying HK activity on hexose-P pools, thereby avoiding the risk of Glc-accelerated death by a stall in glycolytic activity. Secondly, the HK-yielded metabolite G6P is a key regulator of primary metabolism in plants. In particular, it is known to inhibit a SNF1-related protein kinase 1 (SnRK1) (Toroser *et al.*, 2000) and to activate PEPC in plants (Chollet and Vidal, 1996). These enzymes control important aspects of carbon and nitrogen metabolism. The metabolite profiles of the transgenic roots suggest that their carbon and nitrogen metabolisms were not deeply

affected by the manipulation of HK (Figs. 4.4–4.6). Therefore, the Glc/Glc-P and Suc cycles may have efficiently dampened HK activity variations in the root clones. However, the dissipation of ATP by substrate cycling in the sense clones may have led to down-regulation of several processes to reduce ATP expenditure (Fig. 4.6b,c,d,i,o), with possible adverse effects on growth (Fig. 4.3). It thus appears that, at least in roots, variations in HK activity affect cycling fluxes in the upper part of glycolysis, but alone cannot dramatically alter glycolytic flux beyond the hexose-P pool. Our results agree with findings from previous investigations showing that the manipulation of HK activity in potato tubers had limited impact on tuber metabolism (Veramendi *et al.*, 1999; Veramendi *et al.*, 2002). They contrast with greater pools of several amino acids in tomato fruit overexpressing HK, although differences with the wild-type diminished at further stages of development (Roessner-Tunali *et al.*, 2003; Menu *et al.*, 2004). Importantly, ATP levels were decreased in the HK overexpressors regardless of the developmental stage (Roessner-Tunali *et al.*, 2003; Menu *et al.*, 2004). Since Suc cycles are known to occur in tomato fruit (Nguyen-Quoc and Foyer, 2001), it is possible that HK overexpression led to sustained Suc cycling in the transgenic tomato fruit. The associated cost in ATP would be a plausible cause of reduced fruit size and organ-specific effects on downstream metabolism that would need to be explored (Menu *et al.*, 2004). Altogether, our findings suggest that functional Suc and Glc/Glc-P cycles are crucial for adequate glycolytic regulation and growth. Combined Suc and Glc/Glc-P cycling may be the plant alternative to T6P-, G6P- and GKRP inhibitions of yeast and mammalian HKs, respectively, for avoidance of Glc-accelerated death by P_i sequestration in hexose-Ps (Teusink *et al.*, 1998; Iynedjian, 1998). Suc and Glc/Glc-P cycles may fulfill another primordial role in plants by adjusting levels of potent regulatory glycolytic intermediates, such as G6P, to hexose supply and downstream metabolic activity. Our claim of these substrate cycles being key to glycolytic regulation would provide a likely explanation for their sustained activity under various growth conditions including hypoxic stress (Roscher *et al.*, 1998; Rontein *et al.*, 2002).

4.7 Conclusion

In the present study, we have manipulated the expression of HK in a heterotrophic organ. The protein encoded by the *ScHK2* cDNA used to transform the potato roots was previously expressed as a recombinant protein and kinetically characterized (Claeyssen *et al.*, 2006). While several *ScHK2* antisense constructs were used, HK activity could not be reduced by more than 30% in the antisense clones. The overexpression strategy yielded sense clones that contained up to 8 times more HK activity than control clones. We showed by manipulating HK activity levels in the potato roots that HK exerts major control on growth of this heterotrophic organ. Although sugar sensing by HK may have been affected in the transgenics, changes in root growth and metabolite pools were significantly correlated to varying HK activity levels. The physiological relevance of the catalytic function of HK to root growth and metabolism could be explained in terms of glycolytic flux control. Indeed, the FCC of HK over glycolysis at the step of Glc phosphorylation was measured for the first time in a plant tissue, and reached 0.76. Such a high FCC and the growth phenotype of the root clones now bring solid evidence for the long-claimed importance of HK in plant primary metabolism (Renz and Stitt, 1993; Claeyssen and Rivoal, 2007). Importantly, the FCC of HK over Glc influx is comparably high in mammals and plants, despite contrasting differences in the structure and regulation of their glycolytic pathways. This points to HK as a universal control point at the gate of glycolysis, although this enzyme may be involved in different regulatory mechanisms among Eukaryotes (Iynedjian, 1998). There was evidence to suggest that in the potato roots, HK may be implicated in glycolytic regulation via Glc/Glc-P and Suc cycling. We propose that these substrate cycles are necessary to adjust G6P levels to hexose supply and downstream glycolytic activity, and to avoid a stall in glycolytic activity due to sequestration of P_i in hexose-Ps. Elucidating the mechanisms underlying the regulation of the Suc and Glc/Glc-P cycles will probably help gain further insight into their functions and into the regulation of plant glycolysis.

4.8 Acknowledgements

We are indebted to Stéphane Daigle for helpful advice on statistical analyses. We also thank Les Semences Élite du Québec (Les Buissons, QC, Canada) for providing seed potatoes used in this study.

4.9 References

Alonso, A.P., Vigeolas, H., Raymond, P., Rolin, D., Dieuaide-Noubhani, M., 2005. A new substrate cycle in plants. Evidence for a high glucose-phosphate-to-glucose turnover from *in vivo* steady-state and pulse-labeling experiments with [^{13}C]glucose and [^{14}C]glucose. Plant Physiol. 138, 2220-2232.

An, G., Ebert, P.R., Mitra, A., Ha, S.B., 1988. Binary vectors. In: *Plant Molecular Biology Manual.* Dordrecht: Kluwer Academic Publisher, pp.1-19.

Aon, M.A., Cortassa, S., 1998. Catabolite repression mutants of *Saccharomyces cerevisiae* show altered fermentative metabolism as well as cell cycle behavior in glucose-limited chemostat cultures. Biotechnol. Bioeng. 59, 203-213.

Ap Rees, T., Hill, S.A., 1994. Metabolic control analysis of plant metabolism. Plant Cell Environ. 17, 587-599.

Appeldoorn, N.J.G., de Bruijn, S.M., Koot-Gronsveld, E.A.M., Visser, R.G.F., Vreugdenhil, D., van der Plas, L.H.W., 1997. Developmental changes of enzymes involved in conversion of sucrose to hexose-phosphate during early tuberisation of potato. Planta 202, 220-226.

Azevedo, R.A., Arruda, P., Turner, W.L., Lea, P.J., 1997. The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochem. 46, 395-419.

Borchert, S., Harborth, J., Schunemann, D., Hoferichter, P., Heldt, H.W., 1993. Studies of the enzymic capacities and transport properties of pea root plastids. Plant Physiol. 101, 303-312.

Bouché, N., Fait, A., Zik, M., Fromm, H., 2004. The root-specific glutamate decarboxylase (GAD1) is essential for sustaining GABA levels in *Arabidopsis*. Plant Mol. Biol. 55, 315-325.

Bouny, J.M., Saglio, P.H., 1996. Glycolytic flux and hexokinase activities in anoxic maize root tips acclimated by hypoxic pretreatment. Plant Physiol. 111, 187-194.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Canvin, D.T., Beevers, H., 1961. Sucrose synthesis from acetate in the germinating castor bean: Kinetics and pathway. J. Biol. Chem. 236, 988-995.

Carswell, C., Grant, B.R., Theodorou, M.E., Harris, J., Niere, J.O., Plaxton, W.C., 1996. The fungicide Phosphonate disrupts the phosphate-starvation response in *Brassica nigra* seedlings. Plant Physiol. 110, 105-110.

Chollet, R., Vidal, J., 1996. Phopsho*enol*pyruvate carboxylase: A ubiquitous, highly regulated enzyme in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 273-298.

Claeyssen, E., Rivoal, J., 2007. Isozymes of plant hexokinase: Occurrence, properties and functions. Phytochem. 68, 709-731.

Claeyssen, E., Wally, O., Matton, D.P., Morse, D., Rivoal, J., 2006. Cloning, expression, purification, and properties of a putative plasma membrane hexokinase from *Solanum chacoense*. Protein Expr. Purif. 47, 329-339.

Dai, N., Schaffer, A., Petreikov, M., Shahak, Y., Giller, Y., Ratner, K., Levine, A., Granot, D., 1999. Overexpression of *Arabidopsis* hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. Plant Cell 11, 1253-1266.

Dieuaide-Noubhani, M., Raffard, G., Canioni, P., Pradet, A., Raymond, P., 1995. Quantification of compartmented metabolic fluxes in maize root tips using isotope distribution from ^{13}C- or ^{14}C-labeled glucose. J. Biol. Chem. 270, 13147-13159.

Dorion, S., Lalonde, S., Saini, H.S., 1996. Induction of male sterility in wheat by meiotic-state water deficit is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. Plant Physiol. 111, 137-145.

Dorion, S., Parveen, Jeukens, J., Matton, D.P., Rivoal, J., 2005. Cloning and characterization of a cytosolic isoform of triosephosphate isomerase developmentally regulated in potato leaves. Plant Sci. 168, 183-194.

Duff, S.M.G., Moorhead, G.B., Lefebvre, D.D., Plaxton, W.C., 1989. Phosphate starvation inducible 'bypasses' of adenylate and phosphate dependent glycolytic enzymes in *Brassica nigra* suspension cells. Plant Physiol. 90, 1275-1278.

Ernandes, J.R., De Meirsman, C., Rolland, F., Winderickx, J., De Winde, J., Brandao, R.L., Thevelein, J.M., 1998. During the initiation of fermentation overexpression of hexokinase PII in yeast transiently causes a similar deregulation of glycolysis as deletion of Tps1. Yeast 14, 255-269.

Fell, D.A., 1992. Metabolic control analysis: A survey of its theoretical and experimental development. Biochem. J. 286, 313-330.

Fernie, A.R., Roscher, A., Ratcliffe, R.G., Kruger, N.J., 2001. Fructose-2,6-bisphosphate activates pyrophosphate:fructose-6-phosphate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. Planta 212, 250-263.

Fernie, A.R., Tiessen, A., Stitt, M., Willmitzer, L., Geigenberger, P., 2002. Altered metabolic fluxes result from shifts in metabolite levels in sucrose phosphorylase-expressing potato tubers. Plant Cell Environ. 25, 1219-1232.

Freixes, S., Thibaud, M.-C., Tardieu, F., Müller, B., 2002. Root elongation and branching is related to local hexose concentration in *Arabidopsis thaliana* seedlings. Plant Cell Environ. 25, 1357-1366.

Frömmer, W.B., Schulze, W.X., Lalonde, S., 2003. Hexokinase, Jack-of-all-trades. Science 300, 261-263.

Gauthier, D.A., Turpin, D.H., 1994. Inorganic phosphate (P_i) enhancement of dark respiration in the P_i-limited green alga *Selenastrum minutum* (interactions between H^+/P_i cotransport, the plasmalemma H^+-ATPase, and dark respiratory carbon flow). Plant Physiol. 104, 629-637.

Grossbard, L., Schimke, R.T., 1966. Multiple hexokinases of rat tissues. Purification and comparison of soluble forms. J. Biol. Chem. 241, 3546-3560.

Halford, N.G., Purcell, P.C., Hardie, D.G., 1999. Is hexokinase really a sugar sensor in plants? Trends Plant Sci. 4, 117-120.

Heinrich, R., Rapoport, T.A., 1974. A linear steady-state treatment of enzymatic chains. General properties, control and effector strength. Eur. J. Biochem. 42, 89-95.

Herrmann, K.M., Weaver, L.M., 1999. The shikimate pathway. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 473-503.

Hodgson, R.J., Plaxton, W.C., 1998. Purification and characterization of cytosolic fructose-1,6 bisphosphate aldolase from endosperm of germinated castor oil seeds. Arch. Biochem. Biophys. 355, 189-196.

Iynedjian, P.B., 1998. Glycolysis, turbo design and the endocrine pancreatic β cell. Trends Biochem. Sci. 23, 467-468.

Jang, J.C., Leon, P., Zhou, L., Sheen, J., 1997. Hexokinase as a sugar sensor in higher plants. Plant Cell 9, 5-19.

Joly, S., Bruneau, A., 2006. Incorporating allelic variation for reconstructing the evolutionary history of organisms from multiple genes: An example from *Rosa* in North America. Syst. Biol. 55, 623-636.

Journet, E.P., Bligny, R., Douce, R., 1986. Biochemical changes during sucrose deprivation in higher plant cells. J. Biol. Chem. 261, 3193-3199.

Junker, B.H., Wuttke, R., Nunes-Nesi, A., Steinhauser, D., Schauer, N., Bussis, D., Willmitzer, L., Fernie, A.R., 2006. Enhancing vacuolar sucrose cleavage within the developing potato tuber has only minor effects on metabolism. Plant Cell Physiol. 47, 277-289.

Kacser, H., Burns, J.A., 1973. The control of flux. Symp. Soc. Exp. Biol. 27, 65-104.

Kashiwaya, Y., Sato, K., Tsuchiya, N., Thomas, S., Fell, D.A., Veech, R.L., Passonneau, J.V., 1994. Control of glucose utilization in working perfused rat heart. J. Biol. Chem. 269, 25502-25514.

Kim, J.Y., Mahe, A., Brangeon, J., Prioul, J.-L., 2000. A maize vacuolar invertase, IVR2, is induced by water stress. Organ/tissue specificity and diurnal modulation of expression. Plant Physiol. 124, 71-84.

Klein, D., Stitt, M., 1998. Effects of 2-deoxyglucose on the expression of *rbcS* and the metabolism of *Chenopodium rubrum* cell-suspension cultures. Planta 205, 223-234.

Koebmann, B.J., Westerhoff, H.V., Snoep, J.L., Nilsson, D., Jensen, P.R., 2002. The glycolytic flux in *Escherichia coli* is controlled by the demand for ATP. J. Bacteriol. 184, 3909-3916.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Larsson, C., Pahlman, I.L., Gustafsson, L., 2000. The importance of ATP as a regulator of glycolytic flux in *Saccharomyces cerevisiae*. Yeast 16, 797-809.

Lea, P.J., Miflin, B.J., 2003. Glutamate synthase and the synthesis of glutamate in plants. Plant Physiol. Biochem. 41, 555-564.

Liu, L.M., Li, Y., Du, G.C., Chen, J., 2006. Increasing glycolytic flux in *Torulopsis glabrata* by redirecting ATP production from oxidative phosphorylation to substrate-level phosphorylation. J. Appl. Microbiol. 100, 1043-1053.

Meléndez-Hevia, E., Mateo, F., Torres, N.V., 1992. Control analysis of rat liver glycolysis under different glucose concentrations. The substrate approach and the role of glucokinase. Mol. Cell Biochem. 115, 1-9.

Menu, T., Saglio, P., Granot, D., Dai, N., Raymond, P., Ricard, B., 2004. High hexokinase activity in tomato fruit perturbs carbon and energy metabolism and reduces fruit and seed size. Plant Cell Environ. 27, 89-98.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Liu, Y.X., Hwang, I., Jones, T., Sheen, J., 2003. Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science 300, 332-336.

Moore, L., Warren, G., Strobel, G., 1979. Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. Plasmid 2, 617-626.

Moorhead, G.B., Hodgson, R.J., Plaxton, W.C., 1994. Copurification of cytosolic fructose-1,6-bisphosphatase and cytosolic aldolase from endosperm of germinating castor oil seeds. Arch. Biochem. Biophys. 312, 326-335.

Moraes, T.F., Plaxton, W.C., 2000. Purification and characterization of phosphoenolpyruvate carboxylase from *Brassica napus* (rapeseed) suspension cell cultures: Implications for phospho*enol*pyruvate carboxylase regulation during phosphate starvation, and the integration of glycolysis with nitrogen assimilation. Eur. J. Biochem. 267, 4465-4476.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

Nguyen-Quoc, B., Foyer, C.H., 2001. A role for 'futile cycles' involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. J. Exp. Bot. 52, 881-889.

Petit, A., David, C., Dahl, G.A., Ellis, J.G., Guyon, P., Casseldelbart, F., Tempe, J., 1983. Further extension of the opine concept. Plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. Mol. Gen. Genet. 190, 204-214.

Plaxton, W.C., 1990. Glycolysis. In: *Methods in Plant Biochemistry.* Academic Press Limited, pp. 145-173.

Plaxton, W.C., 1996. The organization and regulation of plant glycolysis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 185-214.

Plaxton, W.C., Smith, C.R., Knowles, V.L., 2002. Molecular and regulatory properties of leucoplast pyruvate kinase from *Brassica napus* (rapeseed) suspension cells. Arch. Biochem. Biophys. 400, 54-62.

Puigjaner, J., Rais, B., Burgos, M., Comin, B., Ovadi, J., Cascante, M., 1997. Comparison of control analysis data using different approaches: Modelling and experiments with muscle extract. FEBS Lett. 418, 47-52.

Rapoport, T.A., Heinrich, R., Jacobasch, G., Rapoport, S.M., 1974. A linear steady-state treatment of enzymatic chains. A mathematical model of glycolysis of human erythrocytes. Eur. J. Biochem. 42, 107-120.

Renz, A., Merlo, L., Stitt, M., 1993. Partial purification from potato tubers of three fructokinases and three hexokinases which show differing organ and developmental specificity. Planta 190, 156-165.

Renz, A., Stitt, M., 1993. Substrate specificity and product inhibition of different forms of fructokinases and hexokinases in developing potato tubers. Planta 190, 166-175.

Rivoal, J., Hanson, A.D., 1994. Metabolic control of anaerobic glycolysis - Overexpression of lactate dehydrogenase in transgenic tomato roots supports the Davies-Roberts hypothesis and points to a critical role for lactate secretion. Plant Physiol. 106, 1179-1185.

Rivoal, J., Ricard, B., Pradet, A., 1989. Glycolytic and fermentative enzyme induction during anaerobiosis in rice seedlings. Plant Physiol. Biochem. 27, 43-52.

Roessner-Tunali, U., Hegemann, B., Lytovchenko, A., Carrari, F., Bruedigam, C., Granot, D., Fernie, A.R., 2003. Metabolic profiling of transgenic tomato plants

overexpressing hexokinase reveals that the influence of hexose phosphorylation diminishes during fruit development. Plant Physiol. 133, 84-99.

Rohwer, J.M., Botha, F.C., 2001. Analysis of sucrose accumulation in the sugar cane culm on the basis of in vitro kinetic data. Biochem. J. 358, 437-445.

Rolland, F., Baena-Gonzalez, E., Sheen, J., 2006. Sugar sensing and signaling in plants: Conserved and novel mechanisms. Annu. Rev. Plant Biol. 57, 675-709.

Rolland, F., Winderickx, J., Thevelein, J.M., 2001. Glucose-sensing mechanisms in eukaryotic cells. Trends Biochem. Sci. 26, 310-317.

Rontein, D., Dieuaide-Noubhani, M., Dufourc, E.J., Raymond, P., Rolin, D., 2002. The metabolic architecture of plant cells. Stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. J. Biol. Chem. 277, 43948-43960.

Roscher, A., Emsley, L., Raymond, P., Roby, C., 1998. Unidirectional steady state rates of central metabolism enzymes measured simultaneously in a living plant tissue. J. Biol. Chem. 273, 25053-25061.

Salon, C., Raymond, P., Pradet, A., 1988. Quantification of carbon fluxes through the tricarboxylic acid cycle in early germinating lettuce embryos. J. Biol. Chem. 263, 12278-12287.

Sambrook, J., Russell, D.W., 2001. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sander, A., Krausgrill, S., Greiner, S., Weil, M., Rausch, T., 1996. Sucrose protects cell wall invertase but not vacuolar invertase against proteinaceous inhibitors. FEBS Lett. 385, 171-175.

Scheibe, R., 2004. Malate valves to balance cellular energy supply. Physiol. Plant. 120, 21-26.

Shelp, B.J., Bown, A.W., McLean, M.D., 1999. Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. 4, 446-452.

Slocum, R.D., 2005. Genes, enzymes and regulation of arginine biosynthesis in plants. Plant Physiol. Biochem. 43, 729-745.

Sung, S.-J.S., Xu, D.-P., Black, C.C., 1989. Identification of actively filling sucrose sinks. Plant Physiol. 89, 1117-1121.

Sweet, I.R., Matschinsky, F.M., 1995. Mathematical model of β-cell glucose metabolism and insulin release. I. Glucokinase as glucosensor hypothesis. Am. J. Physiol. 268, E775-E788.

Sweetlove, L.J., Burrell, M.M., Ap Rees, T., 1996. Characterization of transgenic potato (*Solanum tuberosum*) tubers with increased ADPglucose pyrophosphorylase. Biochem. J. 320, 487-492.

Sweetlove, L.J., Tomlinson, K.L., Hill, S.A., 2002. The effect of exogenous sugars on the control of flux by adenosine 5'-diphosphoglucose pyrophosphorylase in potato tuber discs. Planta 214, 741-750.

Tang, G.Q., Luscher, M., Sturm, A., 1999. Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. Plant Cell 11, 177-190.

Tang, G.Q., Sturm, A., 1999. Antisense repression of sucrose synthase in carrot (*Daucus carota* L.) affects growth rather than sucrose partitioning. Plant Mol. Biol. 41, 465-479.

Tesfaye, M., Temple, S.J., Allan, D.L., Vance, C.P., Samac, D.A., 2001. Overexpression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminum. Plant Physiol. 127, 1836-1844.

Teusink, B., Walsh, M.C., van Dam, K., Westerhoff, H.V., 1998. The danger of metabolic pathways with turbo design. Trends Biochem. Sci. 23, 162-169.

Thevelein, J.M., Hohmann, S., 1995. Trehalose synthase: guard to the gate of glycolysis in yeast? Trends Biochem. Sci. 20, 3-10.

Thomas, S., Fell, D.A., 1998. A control analysis exploration of the role of ATP utilisation in glycolytic-flux control and glycolytic-metabolite-concentration regulation. Eur. J. Biochem. 258, 956-967.

Toroser, D., Plaut, Z., Huber, S.C., 2000. Regulation of a plant SNF1-related protein kinase by glucose-6-phosphate. Plant Physiol. 123, 403-412.

Urbanczyk-Wochniak, E., Leisse, A., Roessner-Tunali, U., Lytovchenko, A., Reismeier, J., Willmitzer, L., Fernie, A.R., 2003. Expression of a bacterial xylose isomerase in potato tubers results in an altered hexose composition and a consequent induction of metabolism. Plant Cell Physiol. 44, 1359-1367.

Utriainen, T., Lovisatti, S., Makimattila, S., Bertoldo, A., Weintraub, S., DeFronzo, R., Cobelli, C., Yki-Järvinen, H., 2000. Direct measurement of the lumped constant for 2-deoxy-[1-^{14}C]glucose *in vivo* in human skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 279, E228-E233.

Veramendi, J., Fernie, A.R., Leisse, A., Willmitzer, L., Trethewey, R.N., 2002. Potato hexokinase 2 complements transgenic Arabidopsis plants deficient in hexokinase 1 but does not play a key role in tuber carbohydrate metabolism. Plant Mol. Biol. 49, 491-501.

Veramendi, J., Roessner, U., Renz, A., Willmitzer, L., Trethewey, R.N., 1999. Antisense repression of hexokinase 1 leads to an overaccumulation of starch in leaves of transgenic potato plants but not to significant changes in tuber carbohydrate metabolism. Plant Physiol. 121, 123-134.

Wang, H., Iynedjian, P.B., 1997. Modulation of glucose responsiveness of insulinoma β-cells by graded overexpression of a glucokinase. Proc. Natl. Acad. Sci. USA 94, 4372-4377.

Wilson, J.E., 2003. Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. J. Exp. Biol. 206, 2049-2057.

Wood, S.M., King, S.P., Kuzma, M.M., Blakeley, S.D., Newcomb, W., Dennis, D.T., 2002. Pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase overexpression in transgenic tobacco: Physiological and biochemical analysis of source and sink tissues. Can. J. Bot. 80, 983-992.

Chapitre 5.

Discussion Générale et Conclusions

5.1 Discussion générale

La revue de littérature (chapitre 2) et les deux articles de recherche (chapitres 3 et 4) présentés dans cette thèse nous ont permis de progresser dans notre compréhension de la fonction catalytique de l'HK végétale. Nous discutons ici des points majeurs de nos recherches.

5.1.1 La localisation subcellulaire fait partie intégrante des modes d'action des isoformes HK

L'un des principaux arguments de notre revue de littérature est que la localisation subcellulaire des isoformes HK sert leurs rôles individuels et spécifiques. Cet argument est conforté par deux études, parues trop tard pour être intégrées à la revue, et qui sont donc décrites plus amplement ici.

5.1.1.1 Importance de la localisation nucléaire de l'HK dans sa fonction de perception d'hexoses chez A. thaliana

Dans une publication récente, l'équipe de J. Sheen a clarifié le mode d'action de l'isoforme AtHXK1 d'*Arabidopsis* dans la perception et la transduction du signal Glc (Cho *et al.*, 2006b). Ces auteurs ont montré que dans le noyau, AtHXK1 interagissait avec l'H^+-ATPase vacuolaire B1 (VHA-B1) et la sous-unité RPT5B de la particule régulatrice 19S du protéasome. Le complexe ainsi formé se liait aux promoteurs des gènes photosynthétiques *CAB2* et *CAB3* (qui codent pour les protéines 2 et 3 de liaison à la chlorophylle *a/b*), et assurait leur répression par le Glc sans métaboliser ce dernier (Cho *et al.*, 2006b). Ces résultats confirment donc que la présence d'AtHXK1 dans le noyau est indispensable à son rôle d'isoforme dans la fonction de perception et de signalisation du Glc. Rappelons qu'AtHXK1 a également été localisée sur la face cytosolique de la membrane externe mitochondriale (Giegé *et al.*, 2003). Il a été suggéré, mais non démontré, que cette localisation membranaire a une signification physiologique dans la fonction de perception des hexoses (Balasubramanian *et al.*, 2007). Si tel est le cas, les recherches futures

permettront de comprendre quel aspect de cette fonction requiert la présence d'AtHXK1 à la surface des mitochondries. Il importera aussi d'établir s'il y a translocation d'AtHXK1 entre la membrane mitochondriale et le noyau, ou si une partie de la population de protéines AtHXK1 reste en permanence dans le noyau pour y accomplir la signalisation des hexoses. Assurément, l'investigation des mécanismes responsables de la localisation subcellulaire des isoformes HK permettra de clarifier les modes d'action de celles-ci dans leurs rôles individuels. Par ailleurs, les partenaires d'interaction nucléaires identifiés à ce jour pour les senseurs de Glc HK PII de levure et HK IV de mammifère sont sensiblement différents de ceux d'AtHXK1 chez *Arabidopsis* (Rolland *et al.*, 2001; Cho *et al.*, 2006b). Cependant, des sous-unités RPT de la particule 19S du protéasome ont été impliquées dans le contrôle de la transcription chez la levure (Gonzalez *et al.*, 2002; Lee *et al.*, 2005). Il serait donc intéressant de vérifier si les orthologues de RPT5B peuvent avoir un rôle similaire dans la transduction du signal Glc chez la levure et les mammifères (Cho *et al.*, 2006b).

5.1.1.2 Importance de la localisation subcellulaire de l'HK dans sa fonction de régulation de la mort cellulaire programmée chez N. benthamiana

L'implication de l'HK dans la mort cellulaire programmée (MCP) a été démontrée et avec elle, la signification physiologique de son association à la membrane mitochondriale externe (Kim *et al.*, 2006). La sousexpression de l'isoforme NbHXK1 dans des plants de *Nicotiana benthamiana* entraînait l'apparition dans les feuilles de marqueurs anatomiques et cellulaires de la MCP. De plus, des mitochondries purifiées et exposées à des doses de H_2O_2 déclenchant la MCP avaient un phénotype de MCP moins prononcé en présence de NbHXK1 (Kim *et al.*, 2006). Ces auteurs ont donc révélé une nouvelle fonction pour l'HK végétale dans la régulation de la MCP. Chez les animaux, l'apoptose débute dans la mitochondrie avec la fuite de solutés et du cytochrome *c*, et la perte de son potentiel membranaire (Gulbins *et al.*, 2003). Ces étapes résultent de l'ouverture du pore de transition de perméabilité (PTP) situé aux points de contact entre les membranes interne et externe de la mitochondrie (Gulbins *et al.*, 2003). La fixation inhibée par le G6P de l'HK I ou II de mammifère au canal à anions voltage-dépendant (VDAC), un composant du PTP, entraîne sa fermeture et empêche l'apoptose (Azoulay-Zohar *et al.*, 2004; Pastorino *et al.*, 2005). Il

faut noter alors que le VDAC, la perte de potentiel membranaire et la fuite du cytochrome *c* sont des éléments conservés de l'apoptose animale et de la MCP des plantes (Godbole *et al.*, 2003; Yao *et al.*, 2004; Lin *et al.*, 2006). Il a été démontré chez *Arabidopsis* qu'AtHXK1 pouvait se lier *in vivo* aux deux isoformes VDAC les plus abondantes de la membrane mitochondriale (Balasubramanian *et al.*, 2007). Chez *N. benthamiana*, par contre, le site d'interaction de la membrane mitochondriale externe avec NbHXK1 n'a pas été identifié (Kim *et al.*, 2006). De plus, des composés dissociant HK I et II de la mitochondrie chez les mammifères sont restés sans effet sur les HKs associées aux mitochondries de riz, de maïs et de pois (*Pisum sativum*), suggérant des mécanismes d'interaction différents chez les plantes (Dry *et al.*, 1983; Tanner *et al.*, 1983; Rezende *et al.*, 2006; Balasubramanian *et al.*, 2007). Néanmoins, Kim *et al.* (2006) ont prouvé que l'extrémité N-terminale de NbHXK1 était indispensable à son association à la mitochondrie et à l'inhibition de la fuite du cytochrome *c*. Ces auteurs ont donc démontré que la localisation de NbHXK1 sur la face cytosolique de la membrane mitochondriale externe faisait partie intégrante de son mode d'action dans la fonction de régulation de la MCP.

La fonction de régulation de la MCP a été explorée dans des plants d'*Arabidopsis* surexprimant AtHXK1 ou AtHXK2, deux isoformes associées à la membrane mitochondriale (Giegé *et al.*, 2003; Kim *et al.*, 2006). Les transformants présentaient un phénotype de MCP moins marqué que les contrôles lors d'un traitement avec des agents pro-apoptotiques (Kim *et al.*, 2006). L'étude d'AtHXK1 a donc établi que cette isoforme peut exercer une fonction de régulation de la MCP au niveau de la membrane mitochondriale, en plus de celle de perception et de signalisation du Glc dans le noyau (Cho *et al.*, 2006b; Kim *et al.*, 2006). Il importe alors de déterminer si une translocation d'AtHXK1 peut avoir lieu entre la membrane mitochondriale et le noyau, et contribuer à la régulation de l'une ou l'autre des fonctions dans la MCP et la signalisation d'hexoses. La pluralité de localisations et fonctions d'AtHXK1 présente des similitudes avec celle d'HK IV chez les mammifères, dont la fonction varie selon qu'elle se trouve dans le noyau, le cytosol ou à la surface des mitochondries (Lange, 2007). Dans le foie, la hausse du niveau de Glc cellulaire dissocie le complexe HK IV-GKRP nucléaire, ce qui active HK IV et permet sa translocation vers le cytosol où elle assure sa double fonction catalytique et de

senseur de Glc (Baltrusch et Tiedge, 2006; Shin *et al.*, 2007). Une partie de la population de protéines HK IV reste en permanence dans le noyau, possiblement pour y exercer une fonction non-catalytique (Lange, 2007). Dans le foie, HK IV est également présente sur la face cytosolique de la membrane mitochondriale externe, où elle coordonne le métabolisme du Glc et l'apoptose (Danial *et al.*, 2003). Ces similitudes laissent supposer que la clarification des fonctions et des mécanismes de ciblage cellulaire ou de translocation d'HK IV inspirera les recherches sur AtHXK1 et sur d'autres HKs végétales.

Les études récentes de l'HK végétale ont donc révélé deux nouvelles fonctions en plus de sa fonction catalytique dans la glycolyse, tout en établissant la signification physiologique de la localisation des isoformes impliquées. La fonction de l'HK dans la perception et la signalisation d'hexoses repose sur son interaction avec plusieurs partenaires dans le noyau pour une régulation transcriptionnelle de gènes sensibles aux hexoses (Cho *et al.*, 2006b). L'autre fonction de l'HK concerne la régulation de la cascade d'évènements constituant la MCP, et nécessite qu'elle soit présente sur la face cytosolique de la membrane mitochondriale externe (Kim *et al.*, 2006). Il ressort que par ces deux fonctions, l'HK végétale joue un rôle crucial dans la vie de la plante en intégrant la signalisation et le métabolisme du Glc à d'autres voies de signalisation et à la MCP, respectivement (Moore *et al.*, 2003; Cho *et al.*, 2006b; Kim *et al.*, 2006). Ces études confirment l'hypothèse souvent évoquée d'une implication de la localisation subcellulaire des isoformes HK dans leurs rôles spécifiques (Wiese *et al.*, 1999; da-Silva *et al.*, 2001; Olsson *et al.*, 2003; Claeyssen et Rivoal, 2007). Il sera intéressant de déterminer si la présence de l'HK végétale à la surface de la membrane mitochondriale externe lui permet de coordonner les activités glycolytique et respiratoire et la MCP comme chez les mammifères (Kim et Dang, 2005). Enfin, l'étude de l'isoforme AtHXK1 d'*Arabidopsis* tend à montrer qu'une même isoforme peut remplir l'une ou l'autre de ces nouvelles fonctions selon sa localisation subcellulaire (Cho *et al.*, 2006b; Kim *et al.*, 2006). La question de la contribution des isoformes HK de la cellule aux différentes fonctions reste donc entière, et nécessitera d'être investiguée dans les futures études d'HKs végétales.

5.1.2 Approches systémique et réductionniste dans l'étude de la fonction catalytique de l'HK

La présente thèse est consacrée à l'étude de la fonction catalytique de l'HK, notamment de l'importance de l'HK dans la régulation de la glycolyse et dans le contrôle de son flux. Comme nous l'avons expliqué en Introduction, le contrôle du flux glycolytique est réparti sur les enzymes de la voie, et son analyse doit intégrer l'influence du reste du métabolisme sur l'enzyme considérée. Nous avons donc eu recours à la MCA (chapitre 4) afin de satisfaire aux requis d'une telle logique systémique, selon les termes de Fell (1997). Nous avons adopté en parallèle une approche réductionniste (chapitre 3), à savoir l'étude de l'HK séparément du reste de la glycolyse et ce, pour trois raisons. D'une part, les caractérisations cinétiques d'enzymes glycolytiques ont largement contribué aux connaissances sur la régulation de la voie chez les plantes (Plaxton, 1996). D'autre part, les preuves de l'implication des propriétés cinétiques et localisations subcellulaires des isoformes HK dans leurs rôles spécifiques (chapitre 2) nous ont incités à caractériser l'isoforme ScHK2 utilisée dans ce projet. Enfin, les données sur les propriétés cinétiques et les rôles des isoformes HK sont un support indispensable à l'étude systémique du contrôle de l'HK sur le flux glycolytique. Nous discutons ici des principaux résultats obtenus ainsi que des limites des approches systémique et réductionniste dans l'étude de la fonction catalytique de l'HK végétale.

5.1.2.1 Localisation présumée de ScHK2 à la membrane plasmique

Outre le fait qu'elle soit la première caractérisation cinétique d'une HK de plante purifiée à homogénéité, notre étude a permis d'attribuer à ScHK2 une localisation et un rôle inédits pour une HK végétale (chapitre 3). Toutefois, la présence de ScHK2 sur la face cytosolique de la membrane plasmique a été suggérée sur la base de motifs consensus situés à son extrémité N-terminale (Claeyssen *et al.*, 2006). Or, il a été démontré que l'extrémité N-terminale d'HKs de type B (comme ScHK2) était indispensable à leur insertion ou à leur association à certaines membranes (Wiese *et al.*, 1999; Damari-Weissler *et al.*, 2006; Kim *et al.*, 2006). Cependant, les études protéomiques, d'immunolocalisation, ou de fusion avec

la protéine fluorescente verte (GFP), n'ont jamais localisé d'HKs de type B au niveau de la voie de sécrétion ou de la membrane plasmique (Wiese *et al.*, 1999; Borner *et al.*, 2005; Komatsu *et al.*, 2006; Balasubramanian *et al.*, 2007). Les isoformes AtHXK1–2 d'*A. thaliana*, LeHxk1–3 de tomate, et NbHXK1 de *N. benthamiana*, sont toutes associées à la membrane mitochondriale, AtHXK1 se trouvant aussi en faibles quantités dans le noyau (Giegé *et al.*, 2003; Cho *et al.*, 2006b; Damari-Weissler *et al.*, 2006; Kandel-Kfir *et al.*, 2006; Kim *et al.*, 2006). Quant à SoHxK1 de l'épinard, une étude récente a remis en cause son insertion dans la membrane externe de l'enveloppe chloroplastique, tout en démontrant son association à la mitochondrie (Wiese *et al.*, 1999; Damari-Weissler *et al.*, 2007). Par ailleurs, l'HK n'apparaît pas dans le protéome de la membrane plasmique d'*Arabidopsis* ou du riz, qu'il s'agisse de données en ligne ou publiées (site SUBA, http://www.suba.bcs.uwa.edu.au ; Santoni *et al.*, 1998; Marmagne *et al.*, 2004; Tanaka *et al.*, 2004; Dunkley *et al.*, 2006; Heazlewood *et al.*, 2007). La possibilité que ScHK2 soit associée à la membrane mitochondriale comme les autres HKs de type B plutôt qu'à la membrane plasmique mérite donc d'être considérée. Il se peut néanmoins que ScHK2 régisse l'import d'hexoses, comme nous l'avons proposé (Claeyssen *et al.*, 2006), à des quantités difficilement détectables dans les études protéomiques. Une métabolisation efficace du Glc importé par une HK présente à la membrane plasmique contribuerait alors à la disparition rapide du Glc cytosolique dans les cellules racinaires (Deuschle *et al.*, 2006). Par conséquent, une localisation de ScHK2 par fusion avec la GFP ou par immunolocalisation semble nécessaire pour tirer une conclusion définitive.

5.1.2.2 La fonction catalytique de l'HK semble la seule altérée dans les clones racinaires

Notre étude de la fonction catalytique de l'HK dans les clones racinaires de pomme de terre nécessitait d'examiner si les autres fonctions avaient été perturbées par la transgénèse (chapitre 4). Certaines études de manipulation génétique de l'HK n'ont pu écarter la possibilité que les fonctions catalytique et de senseur d'hexoses étaient toutes deux altérées dans les transformants (Jang *et al.*, 1997; Dai *et al.*, 1999; Halford *et al.*, 1999). Le fait que le métabolisme des hexoses en aval de l'HK puisse participer à leur

signalisation dans la voie glycolytique-dépendante ajoute un degré supplémentaire de complexité à la question (Xiao *et al.*, 2000; Lejay *et al.*, 2003; Claeyssen et Rivoal, 2007). Nos clones racinaires ne présentaient pas de signes évidents de MCP, laissant supposer que cette fonction de l'HK n'était pas altérée chez ces derniers (Claeyssen *et al.*, 2007). Surtout, leurs taux de croissance, d'une part, et leurs niveaux cumulés de Glc, Fru, malate, Arg, GABA, Thr, et Phe, d'autre part, étaient corrélés à leurs niveaux d'activité HK. Il apparaît donc que seule la fonction catalytique de l'HK était perturbée dans les racines transgéniques, selon notre estimation quantitative basée sur des coefficients de corrélation (Claeyssen *et al.*, 2007).

Dans nos clones racinaires, les profils d'isoformes HK représentatifs comportaient deux pics d'activité endogènes 1 et 2 (Fig. 4.2), indiquant l'expression d'au moins deux isoformes dans les racines de *S. tuberosum*. La présence d'un pic 2 majeur d'activité HK dans le clone S111 surexprimant ScHK2 suggère que celle-ci contribuait largement à la fonction catalytique de l'HK, tout au moins dans les clones sens. Notre observation que cette HK présumée membranaire et fortement inhibée par l'ADP et la glucosamine (Claeyssen *et al.*, 2006) soit engagée dans la fonction catalytique de l'HK, contredit l'hypothèse de da-Silva *et al.* (2001). En effet, ces auteurs ont avancé que dans les racines de maïs, l'HK non-cytosolique assurait peut-être la fonction de senseur d'hexoses du fait de sa sensibilité à l'ADP et à la glucosamine. Les données sur la perception et la signalisation d'hexoses chez *Arabidopsis* n'appuient pas non plus cette hypothèse. Cette fonction de l'HK requiert la présence de faibles quantités d'AtHXK1 dans le noyau (Cho *et al.*, 2006b), mais aucun lien n'a encore été établi avec la présence d'AtHXK1 à la membrane mitochondriale. Il ressort que l'étude des propriétés cinétiques et localisations subcellulaires des isoformes HK peut nous renseigner sur leurs rôles spécifiques et leurs modes d'action. Mais il faudra encore étudier les isoformes conjointement, identifier leurs partenaires d'interaction et élucider la régulation de leurs rôles individuels afin de comprendre comment sont orchestrées leurs contributions aux différentes fonctions de l'HK. Dans notre étude, la part du pic 1 dans l'activité HK totale était la plus affectée par la stratégie antisens et celle du pic 2 par la stratégie sens (Fig. 4.2). Ceci suggère que les stratégies sens et antisens ont pu affecter différemment les contributions des isoformes HK aux diverses fonctions de l'HK,

avec un effet notable uniquement dans la fonction catalytique. Une limite de notre approche est qu'elle ne nous permettrait pas de déceler ces éventuelles perturbations dans les contributions des différentes isoformes HK. Nous avons pu moduler et clarifier la fonction catalytique de l'HK sans perturber de façon notable les autres fonctions, mais notre approche n'est pas conçue pour étudier le détail de chaque isoforme HK. Par exemple, notre mesure du FCC de l'HK sur la portion amont de la glycolyse n'indique pas quelles sont les contributions relatives des différentes isoformes HK au contrôle du flux glycolytique (Fig. 4.7). Il est probable qu'une étude discriminante des isoformes HK fournira des informations pertinentes sur les mécanismes de régulation de la glycolyse et du contrôle de son flux. Notamment, connaître et modifier la part de ScHK2 ou d'autres isoformes sensibles à l'ADP dans les cycles du Suc et du Glc/Glc-P générateurs d'ADP, clarifiera la régulation de ces cycles et donc, leur mode de régulation de la glycolyse.

5.1.2.3 Implication de l'HK dans la régulation glycolytique par les cycles du Suc et du Glc/Glc-P

Notre analyse des niveaux de métabolites et du FCC de l'HK sur l'amont de la glycolyse suggère un faible contrôle de l'HK sur le flux glycolytique au-delà de sa propre étape (Figs. 4.4–4.7). Le calcul du FCC de l'HK sur toute la voie glycolytique permettrait de tester cette hypothèse. Ceci implique de mesurer le flux glycolytique dans les clones racinaires, par marquage au [U-^{14}C]Glc et dosage de la radioactivité dans l'amidon, les acides aminés et le CO_2 produits (Rivoal et Hanson, 1993; Sweetlove *et al.*, 1999). Si elle est vérifiée, notre hypothèse appuiera le constat que les enzymes d'une voie métabolique ont généralement de faibles FCCs (Fell, 1997). Dans ce cas, les grands changements de flux requièrent l'activation de nombreuses enzymes dans la voie, l'avantage étant alors que les variations des niveaux d'intermédiaires sont faibles (Fell et Thomas, 1995). Toutefois, une telle hypothèse contraste avec les FCCs élevés d'HKs de mammifères, et suggère de profondes différences dans les mécanismes de contrôle du flux glycolytique entre les plantes et les animaux. Ces différences peuvent résider dans la biochimie des HKs végétales et animales. Notamment, HK IV qui compose plus de 90% de l'activité HK des cellules de pancréas, suit une cinétique sigmoïdale, ce qui la distingue des autres isoformes

animales et des HKs végétales (Wang et Iynedjian, 1997b; Lange, 2007). Une autre particularité de cette isoforme est que son activité et sa localisation sont fortement régulées par la GKRP dans le foie (Lange, 2007). Les isoformes HK I et II dans le cerveau et HK III dans le muscle, quant à elles, sont fortement inhibées par le G6P, ce qui n'est pas le cas de la plupart des HKs végétales (Cárdenas *et al.*, 1998; Wilson, 2003; Claeyssen et Rivoal, 2007). Par ailleurs, le T6P a été impliqué dans le contrôle du flux glycolytique chez la levure du fait qu'il inhibe HK PII, par contre ce composé n'a pas d'effet sur les activités HK végétales (Thevelein et Hohmann, 1995; Claeyssen et Rivoal, 2007). Malgré ces observations, il est probable que dans les cellules végétales et certains types de cellules mammifères, le contrôle du flux glycolytique fasse intervenir plusieurs isoformes HK aux propriétés contrastées. Il est alors difficile d'expliquer les disparités dans les FCCs d'HKs animales et végétales uniquement par les différences de propriétés cinétiques des isoformes HK.

Le FCC est une propriété du système étudié, il est donc pertinent de considérer l'impact que peut avoir la structure de la voie glycolytique sur le contrôle de son flux par les enzymes qui la constituent. L'étape de l'HK dans la glycolyse végétale se distingue par sa connexion aux cycles du Suc et du Glc/Glc-P, ainsi qu'à l'OPPP et à de multiples voies de biosynthèses consommatrices d'hexoses-Ps (Claeyssen et Rivoal, 2007). Ces diverses voies sont absentes chez les autres Eucaryotes, à l'exception d'un cycle du Glc/G6P dans les hépatocytes et d'une voie de biosynthèse du T6P chez la levure (Thevelein et Hohmann, 1995; Iynedjian, 1998). À l'inverse, d'autres voies consommatrices de G6P sont propres aux animaux, comme la voie de biosynthèse du glycogène dans le foie (Iynedjian, 1998). Il est donc possible que ces diverses connexions influencent directement le contrôle de l'HK sur le flux glycolytique et soient à l'origine de FCCs contrastés entre les différents organismes eucaryotes. D'autres caractéristiques de la glycolyse végétale peuvent avoir une incidence sur le contrôle de son flux, comme sa compartimentation et sa structure en réseau de réactions redondantes (Plaxton, 1996). De plus, l'un de ses substrats majeurs est le Suc, dont les niveaux sont finement régulés par le biais des cycles du Suc (Plaxton, 1996; Nguyen-Quoc et Foyer, 2001). Il est possible que ces particularités aient une incidence sur la façon dont le changement de flux impulsé par une variation d'activité HK se propage au

reste de la voie. Enfin, la demande en ATP apparaît comme un moteur du flux glycolytique aussi bien chez les plantes que chez les animaux, les levures et les bactéries (Thomas et Fell, 1998; Larsson *et al.*, 2000; Koebmann *et al.*, 2002; Sweetlove *et al.*, 2002; Liu *et al.*, 2006). Cependant, nos données suggèrent une hausse des flux cycliques consommateurs d'ATP dans les clones sens, mais pas une hausse du flux de la voie glycolytique tout entière (Figs. 4.4–4.8, Tableau 4.2). Il semble donc que des facteurs supplémentaires soient impliqués dans le contrôle du flux glycolytique, qui nécessitent d'être investigués.

D'après nos données sur les niveaux de nombreux métabolites et sur le métabolisme du [U-^{14}C]DOG6P (Figs. 4.4–4.8, Tableau 4.2), nous avons proposé que l'HK est impliquée dans la régulation de la glycolyse via les cycles du Suc et du Glc/Glc-P. Cette hypothèse nécessite d'être testée en mesurant le flux cyclique dans nos clones racinaires. De plus, une étude comparative sur des racines de pomme de terre transformées pour sur- ou sousexprimer une Glc-P phosphatase animale permettrait de vérifier l'effet d'un cycle du Glc/Glc-P et de sa modulation sur la régulation glycolytique. Les cycles du Suc n'existant pas chez les animaux et celui du Glc/Glc-P uniquement dans certains types de cellules mammifères, une régulation glycolytique par ces cycles serait une particularité des végétaux. Leur maintien dans diverses conditions de croissance et malgré leur coût élevé en ATP, et l'impact négatif de leur dérégulation sur la croissance racinaire, suggèrent un rôle central dans la régulation glycolytique (Fig 4.3) (Dieuaide-Noubhani *et al.*, 1995; Roscher *et al.*, 1998; Rontein *et al.*, 2002; Alonso *et al.*, 2005). Il importe donc là-encore de déterminer quelles isoformes HK contribuent aux cycles du Suc et du Glc/Glc-P, et quel impact ont leurs sensibilités à l'ADP sur ces flux cycliques qui, par définition, génèrent de l'ADP. Assurément, l'investigation de leur régulation permettra de clarifier leur mode d'action et donc, de mieux comprendre les mécanismes de régulation de la glycolyse végétale.

5.2 Conclusions et perspectives

Il est désormais établi que l'HK remplit au moins trois fonctions distinctes chez les plantes : une fonction catalytique dans la glycolyse, une autre de régulation de la MCP, et

celle non-catalytique de perception et signalisation des hexoses. Ces fonctions sont assurées dans la cellule par plusieurs isoformes HK qui se distinguent par leurs patrons d'expression, leurs propriétés cinétiques et leurs localisations subcellulaires. Nos recherches suggèrent que leurs propriétés cinétiques et localisations subcellulaires font partie intégrante de leurs modes d'action dans des rôles individuels dédiés aux différentes fonctions de l'HK. Sur la base de ces observations, nous avons caractérisé une version recombinante pure de l'isoforme ScHK2 de *S. chacoense* utilisée dans ce projet, après avoir cloné et séquencé son ADNc. Nous lui avons assigné une localisation à la membrane plasmique et un rôle en lien avec la fonction catalytique de l'HK, sur la base de sa séquence N-terminale et de ses propriétés cinétiques. Nous avons également utilisé l'ADNc de ScHK2 pour modifier par transformation les niveaux d'activité HK de racines de la plante modèle *S. tuberosum*. Seule la fonction catalytique de l'HK a été perturbée de façon notable dans cet organe hétérotrophe, ce qui s'accorde avec le rôle présumé de ScHK2. L'approche transgénique nous a permis de montrer que l'HK joue un rôle majeur dans la régulation de la croissance racinaire. De plus, le FCC de l'HK sur la phosphorylation des hexoses a été quantifié pour la première fois chez les plantes, et sa valeur de 0,76 ± 0,08 indique que l'HK contrôle fortement le flux de carbone à cette étape. Ce FCC étant comparable à ceux d'HKs de plusieurs types de cellules mammifères, cette enzyme apparaît comme un point de contrôle universel à l'entrée des hexoses dans la glycolyse. Toutefois, nos résultats indiquent un fort contrôle de l'HK sur le flux glycolytique à sa propre étape, mais pas forcément sur toute la voie comme chez les mammifères. L'HK semble plutôt impliquée dans la régulation glycolytique en modulant le flux parcourant les cycles du Suc et du Glc/Glc-P auxquels elle prend part. En effet, nos données sur les marquages au [U-^{14}C]DOG et sur les niveaux d'hexoses-Ps suggèrent que ces derniers étaient recyclés via les cycles du Suc et du Glc/Glc-P, à des taux proportionnels aux niveaux d'activité HK dans les clones racinaires. À l'instar d'autres systèmes eucaryotes, ces flux cycliques empêcheraient une hausse excessive du flux glycolytique susceptible de séquestrer le P_i dans les hexoses-Ps et d'épuiser l'ATP en bloquant la voie. Ils permettraient également d'ajuster finement les niveaux de G6P qui régulent l'activité PEPC en aval, coordonnant ainsi l'amont et l'aval de la glycolyse, de même que les métabolismes carboné et azoté. Le coût en ATP associé à la régulation glycolytique par les cycles de substrats expliquerait alors les variations de

croissance et de niveaux de métabolites parmi les clones racinaires. L'ensemble de ces résultats atteste d'un net progrès dans notre compréhension de la fonction catalytique de l'HK chez les plantes. Ceci devrait inspirer d'autres études d'enzymes glycolytiques selon les approches réductionniste et systémique afin de compléter le schéma global de régulation et de contrôle du flux de la glycolyse végétale.

Notre approche a permis de moduler la fonction catalytique de l'HK en vue de sa caractérisation. Néanmoins, l'HK remplit au moins trois fonctions qui nécessitent d'être examinées. Il se peut aussi que d'autres fonctions soient encore inconnues du fait d'interactions entre des partenaires atypiques et des isoformes en quantités non détectées, comme l'était la signalisation du Glc par AtHXK1 dans le noyau chez *Arabidopsis* (Cho *et al.*, 2006b). Il importe donc de poursuivre l'effort de caractérisation des isoformes HK et d'élucider leurs rôles individuels en lien aux diverses fonctions de l'HK. Ceci implique d'identifier leurs gènes codants, de les localiser au niveau subcellulaire, de caractériser leurs propriétés cinétiques et de régulation, et d'analyser leurs rôles à l'aide de mutants d'HK ou de la génétique réverse. De plus, investiguer le ciblage de certaines isoformes HK vers leurs multiples sites subcellulaires, ou leur translocation entre ces sites, permettra de clarifier leurs modes d'action. Surtout, notre compréhension des fonctions de l'HK nécessite désormais de déterminer comment les différentes isoformes de la cellule sont recrutées pour les accomplir. Certaines isoformes HK, comme AtHXK1 chez *Arabidopsis*, peuvent exercer différentes fonctions selon leur localisation subcellulaire. Cependant, ni notre étude ni aucune autre n'a encore permis de mesurer et contrôler les contributions relatives des isoformes à une fonction donnée de l'HK pour en comprendre les mécanismes. L'un des défis des recherches futures sur l'HK végétale sera donc d'étudier les isoformes conjointement, et de comprendre comment leurs rôles individuels sont régulés en lien avec leurs contributions aux fonctions de l'HK. L'étude conjointe des isoformes HK d'un tissu à un stade de développement donné est envisageable avec l'essor d'études comparées de transcriptomes, de protéomes, de métabolomes et de profils enzymatiques. Confronter les données sur les transcriptomes, les protéomes et les profils d'activité HK aidera notamment à établir quelles isoformes HK sont soumises à une régulation posttranscriptionnelle et/ou posttraductionnelle (Claeyssen et Rivoal, 2007). Ceci permettra peut-être aussi de

distinguer les isoformes recrutées pour leur activité phosphorylante et celles engagées dans la fonction non-catalytique de perception des hexoses. Par ailleurs, l'intérêt a été démontré d'étudier la biochimie et la physiologie de l'HK végétale dans le contexte cellulaire, en lien avec les niveaux *in vivo* de ses substrats, produits et effecteurs (Renz et Stitt, 1993; Roessner-Tunali *et al.*, 2003). Les données de métabolomique seront donc utiles aux études futures de l'HK, dont les variations d'activité pourront être confrontées à celles d'effecteurs tels que l'ADP ou le G6P. L'utilisation de nanosenseurs est envisageable dans l'exploration des interrelations entre les rôles des isoformes HK, leurs interactions avec des partenaires, leurs localisations subcellulaires, leurs niveaux d'activité et les niveaux en substrats et effecteurs (Lalonde *et al.*, 2005; Deuschle *et al.*, 2006). Ces approches complémentaires aideront à comprendre comment les isoformes HK de la cellule végétale contribuent aux multiples fonctions de l'HK, et à établir si leurs rôles sont séparés, redondants ou s'ils se chevauchent partiellement. Nous serons alors en mesure d'apprécier toute la complexité des mécanismes qui font de cette enzyme à l'interface de la signalisation des hexoses, du métabolisme primaire et de la MCP, un acteur indispensable de la vie des plantes.

Bibliographie Générale

Affourtit, C., Krab, K., Moore, A.L., 2001. Control of plant mitochondrial respiration. Biochim. Biophys. Acta 1504, 58-69.

Alonso, A.P., Vigeolas, H., Raymond, P., Rolin, D., Dieuaide-Noubhani, M., 2005. A new substrate cycle in plants. Evidence for a high glucose-phosphate-to-glucose turnover from *in vivo* steady-state and pulse-labeling experiments with [^{13}C]glucose and [^{14}C]glucose. Plant Physiol. 138, 2220-2232.

Andrews, D.L., MacAlpine, D.M., Johnson, J.R., Kelley, P.M., Cobb, B.G., Drew, M.C., 1994. Differential induction of mRNAs for the glycolytic and ethanolic fermentative pathways by hypoxia and anoxia in maize seedlings. Plant Physiol. 106, 1575-1582.

Aon, M.A., Cortassa, S., 1998. Catabolite repression mutants of *Saccharomyces cerevisiae* show altered fermentative metabolism as well as cell cycle behavior in glucose-limited chemostat cultures. Biotechnol. Bioeng. 59, 203-213.

Aschi-Smiti, S., Chaïbi, W., Brouquisse, R., Ricard, B., Saglio, P., 2003. Assessment of enzyme induction and aerenchyma formation as mechanisms for flooding tolerance in *Trifolium subterraneum* 'Park'. Ann. Bot. 91, 195-204.

Atkinson, D.E., 1977. Cellular energy metabolism and its regulation. Academic Press, New York, NY, USA.

Azoulay-Zohar, H., Israelson, A., Abu-Hamad, S., Shoshan-Barmatz, V., 2004. In self-defence: Hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. Biochem. J. 377, 347-355.

Balasubramanian, R., Karve, A., Kandasamy, M., Meagher, R.B., Moore, B., 2005. A role for the actin cytoskeleton in hexokinase mediated glucose signaling. XVIth International Conference on *Arabidopsis* Research. TAIR publication 501716579 [Abstract]. TAIR publication 501716579 consultée le 01/07/07, disponible à : http://www.arabidopsis.org/servlets/TairObject?type=publicationetid=501716579

Balasubramanian, R., Karve, A., Kandasamy, M., Meagher, R.B., Moore, B., 2007. A role for F-actin in hexokinase-mediated glucose signaling. Plant Physiol. 145, 1423-34.

Baltrusch, S., Tiedge, M., 2006. Glucokinase regulatory network in pancreatic β-cells and liver. Diabetes 55 (Suppl.), S55-S64.

Baud, S., Graham, I.A., 2006. A spatiotemporal analysis of enzymatic activities associated with carbon metabolism in wild-type and mutant embryos of *Arabidopsis* using *in situ* histochemistry. Plant J. 46, 155-169.

Blackman, F.F., 1905. Optima and limiting factors. Ann. Bot. 19, 281-296.

Borner, G.H.H., Sherrier, D.J., Weimar, T., Michaelson, L.V., Hawkins, N.D., MacAskill, A., Napier, J.A., Beale, M.H., Lilly, K.S., Dupree, P., 2005. Analysis of detergent-resistant membranes in *Arabidopsis*. Evidence for plasma membrane lipid rafts. Plant Physiol. 137, 104-116.

Bouny, J.M., Saglio, P.H., 1996. Glycolytic flux and hexokinase activities in anoxic maize root tips acclimated by hypoxic pretreatment. Plant Physiol. 111, 187-194.

Boyer, J.S., 1982. Plant productivity and the environment. Science 218, 443-448.

Bray, E.A., Bailey-Serres, J., Weretilnyk, E., 2000. Responses to abiotic stresses. In: Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.), *Biochemistry and Molecular Biology of Plants*. Am. Soc. Plant Physiol., Rockville, MA, USA, pp. 1158-1203.

Burrell, M.M., Monney, P.J., Blundy, M., Carter, D., Wilson, F., Green, J., Blundy, K.S., ap Rees, T., 1994. Manipulation of 6-phosphofructokinase in potato tubers. Planta 194, 95-101.

Cárdenas, M.L., Cornish-Bowden, A., Ureta, T., 1998. Evolution and regulatory role of the hexokinases. Biochim. Biophys. Acta 1401, 242-264.

Chang, W.W.P., Huang, L., Shen, M., Webster, C., Burlingame, A.L., Roberts, J.K.M., 2000. Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment, and identification of proteins by mass spectrometry. Plant Physiol. 122, 295-317.

Cho, J.I., Ryoo, N., Ko, S., Lee, S.K., Lee, J., Jung, K.H., Lee, Y.H., Bhoo, S., Winderickx, J., An, G., Hahn, T.R., Jeon, J.S., 2006a. Structure, expression, and functional analysis of the hexokinase gene family in rice (*Oryza sativa* L.). Planta 224, 598-611.

Cho, Y.H., Yoo, S.D., Sheen, J., 2006b. Regulatory functions of nuclear hexokinase1 complex in glucose signaling. Cell 127, 579-589.

Claeyssen, E., Dorion, S., Wally, O.S.D., Clendenning, A., Rivoal, J., 2007. Altering hexokinase activity levels in transgenic potato (*Solanum tuberosum*) roots affects

their growth and glycolytic flux at the step of glucose phosphorylation. Soumis à J. Biol. Chem.

Claeyssen, E., Rivoal, J., 2007. Isozymes of plant hexokinase: Occurrence, properties and functions. Phytochem. 68, 709-731.

Claeyssen, E., Wally, O., Matton, D.P., Morse, D., Rivoal, J., 2006. Cloning, expression, purification, and properties of a putative plasma membrane hexokinase from *Solanum chacoense*. Protein Expr. Purif. 47, 329-339.

Coruzzi, G., Last, R., 2000. Amino acids. In: Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.), *Biochemistry and Molecular Biology of Plants*. Am. Soc. Plant Physiol., Rockville, MA, USA, pp. 358-410.

Croteau, R., Kutchan, T.M., Lewis, N.G., 2000. Natural products (secondary metabolites). In: Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.), *Biochemistry and Molecular Biology of Plants*. Am. Soc.Plant Physiol., Rockville, MA, USA, pp. 1250-1318.

da-Silva, W.S., Rezende, G.L., Galina, A., 2001. Subcellular distribution and kinetic properties of cytosolic and non-cytosolic hexokinases in maize seedling roots: Implications for hexose phosphorylation. J. Exp. Bot. 52, 1191-1201.

Dai, N., Kandel-Kfir, M., Petreikov, M., Hanael, R., Levin, I., Ricard, B., Rothan, C., Schaffer, A.A., Granot, D., 2002. The tomato hexokinase LeHXK1 cloning, mapping, expression pattern and phylogenetic relationships. Plant Sci. 163, 581-590.

Dai, N., Schaffer, A., Petreikov, M., Shahak, Y., Giller, Y., Ratner, K., Levine, A., Granot, D., 1999. Overexpression of *Arabidopsis* hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. Plant Cell 11, 1253-1266.

Damari-Weissler, H., Ginzburg, A., Gidoni, D., Mett, A., Krassovskaya, I., Weber, A.P.M., Belausov, E., Granot, D., 2007. Spinach SoHXK1 is a mitochondria-associated hexokinase. Planta 226, 1053-1058.

Damari-Weissler, H., Kandel-Kfir, M., Gidoni, D., Mett, A., Belausov, E., Granot, D., 2006. Evidence for intracellular spatial separation of hexokinases and fructokinases in tomato plants. Planta 224, 1495-1502.

Danial, N.N., Gramm, C.F., Scorrano, L., Zhang, C.Y., Krauss, S., Ranger, A.M., Datta, S.R., Greenberg, M.E., Licklider, L.J., Lowell, B.B., Gygi, S.P., Korsmeyer, S.J., 2003. BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. Nature 424, 952-956.

Davies, J.M., Poole, R.J., Sanders, R., 1993. The computed free energy change of hydrolysis of inorganic pyrophosphate and ATP: Apparent significance for inorganic-pyrophosphate-driven reactions of intermediary metabolism. Biochim. Biophys. Acta 1141, 29-36.

Dennis, D.T., Blakeley, S.D., 2000. Carbohydrate metabolism. In: Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.), *Biochemistry and Molecular Biology of Plants*. Am. Soc. Plant Physiol., Rockville, MA, USA, pp. 630-675.

Deuschle, K., Chaudhuri, B., Okumoto, S., Lager, I., Lalonde, S., Frömmer, W.B., 2006. Rapid metabolism of glucose detected with FRET glucose nanosensors in epidermal cells and intact roots of *Arabidopsis* RNA-silencing mutants. Plant Cell 18, 2314-2325.

Dieuaide-Noubhani, M., Canioni, P., Raymond, P., 1997. Sugar-starvation-induced changes of carbon metabolism in excised maize root tips. Plant Physiol. 115, 1505-1513.

Dieuaide-Noubhani, M., Raffard, G., Canioni, P., Pradet, A., Raymond, P., 1995. Quantification of compartmented metabolic fluxes in maize root tips using isotope distribution from ^{13}C- or ^{14}C-labeled glucose. J. Biol. Chem. 270, 13147-13159.

Doehlert, D.C., 1989. Separation and characterization of four hexose kinases from developing maize kernels. Plant Physiol. 89, 1042-1048.

Drew, M.C., 1997. Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 223-250.

Dry, I.B., Nash, D., Wiskich, J.T., 1983. The mitochondrial localization of hexokinase in pea leaves. Planta 158, 152-156.

Dunkley, T.P.J., Hester, S., Shadforth, I.P., Runions, J., Weimar, T., Hanton, S.L., Griffin, J.L., Bessant, C., Brandizzi, F., Hawes, C., Watson, R.B., Dupree, P., Lilley, K.S., 2006. Mapping the *Arabidopsis* organelle proteome. Proc. Natl. Acad. Sci. USA 103, 6518-6523.

Ettenhuber, C., Radykewicz, T., Kofer, W., Koop, H.U., Bacher, A., Eisenreich, W., 2005. Metabolic flux analysis in complex isotopolog space. Recycling of glucose in tobacco plants. Phytochem. 66, 323-335.

Fell, D.A., 1997. Understanding the control of metabolism. Portland Press, London, England, pp. 1-301.

Fell, D.A., Thomas, S., 1995. Physiological control of metabolic flux: The requirement for multisite modulation. Biochem. J. 311, 35-39.

Fennoy, S.L., Nong, T., Bailey-Serres, J., 1998. Transcriptional and post-transcriptional processes regulate gene expression in oxygen-deprived roots of maize. Plant J. 15, 727-735.

Fernie, A.R., Carrari, F., Sweetlove, L.J., 2004. Respiratory metabolism: Glycolysis, the TCA cycle and mitochondrial electron transport. Curr. Opin. Plant Biol. 7, 254-261.

Fernie, A.R., Tiessen, A., Stitt, M., Willmitzer, L., Geigenberger, P., 2002. Altered metabolic fluxes result from shifts in metabolite levels in sucrose phosphorylase-expressing potato tubers. Plant Cell Environ. 25, 1219-1232.

Fox, T.C., Green, B.J., Kennedy, R.A., Rumpho, M.E., 1998. Changes in hexokinase activity in *Echinochloa phyllopogon* and *Echinochloa crus-pavonis* in response to abiotic stress. Plant Physiol. 118, 1403-1409.

Fox TC, Mujer CV, Andrews DL, Williams AS, Cobb BG, Kennedy RA, Rumpho ME. 1995. Identification and gene-expression of anaerobically induced enolase in *Echinochloa phyllopogon* and *Echinochloa crus-pavonis*. Plant Physiol. 109, 433-443.

Frömmer, W.B., Schulze, W.X., Lalonde, S., 2003. Hexokinase, Jack-of-all-trades. Science 300, 261-263.

Galina, A., da-Silva, W.S., 2000. Hexokinase activity alters sugar-nucleotide formation in maize root homogenates. Phytochem. 53, 29-37.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D., Bairoch, A., 2003. ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 31, 3784-3788.

Geigenberger, P., 2003. Response of plant metabolism to too little oxygen. Curr. Opin. Plant Biol. 6, 247-256.

Geigenberger, P., Stitt, M., Fernie, A.R., 2004. Metabolic control analysis and regulation of the conversion of sucrose to starch in growing potato tubers. Plant Cell Environ. 27, 655-673.

Gibbs, J., Greenway, H., 2003. Mechanisms of anoxia tolerance in plants. I. Growth, survival, and anaerobic catabolism. Functional Plant Biol. 30, 1-47.

Gibbs, J., Morrell, S., Valdez, A., Setter, T.L., Greenway, H., 2000. Regulation of alcoholic fermentation in coleoptiles of two rice cultivars differing in tolerance to anoxia. J. Exp. Bot. 51, 785-796.

Giegé, P., Heazlewood, J.L., Roessner-Tunali, U., Millar, A.H., Fernie, A.R., Leaver, C.J., Sweetlove, L.J., 2003. Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. Plant Cell 15, 2140-2151.

Givan, C.V., 1999. Evolving concepts in plant glycolysis: Two centuries of progress. Biol. Rev. 74, 277-309.

Glawischnig, E., Gierl, A., Tomas, A., Bacher, A., Eisenreich, W., 2002. Starch biosynthesis and intermediary metabolism in maize kernels. Quantitative analysis of metabolite flux by nuclear magnetic resonance. Plant Physiol. 130, 1717-1727.

Godbole, A., Varghese, J., Sarin, A., Mathew, M.K., 2003. VDAC is a conserved element of death pathways in plant and animal systems. Biochim. Biophys. Acta 1642, 87-96.

Gonzalez, F., Delahodde, A., Kodadek, T., Johnston, S.A., 2002. Recruitment of a 19S proteasome subcomplex to an activated promoter. Science 296, 548-550.

Gottlob-McHugh, S.G., Sangwan, R.S., Blakeley, S.D., Vanlerberghe, G.C., Ko, K., Turpin, D.H., Plaxton, W.C., Miki, B.L., Dennis, D.T., 1992. Normal growth of transgenic tobacco plants in the absence of cytosolic pyruvate kinase. Plant Physiol. 100, 820-825.

Guglielminetti, L., Perata, P., Morita, A., Loreti, E., Yamaguchi, J., Alpi, A., 2000. Characterization of isoforms of hexose kinases in rice embryo. Phytochem. 53, 195-200.

Guglielminetti, L., Perata, P., Alpi, A., 1995. Effect of anoxia on carbohydrate metabolism in rice seedlings. Plant Physiol. 108, 735-741.

Gulbins, E., Dreschers, S., Bock, J., 2003. Role of mitochondria in apoptosis. Exp. Physiol. 88, 85-90.

Hajirezaei, M., Sonnewald, U., Viola, R., Carlisle, S.M., Dennis, D.T., Stitt, M., 1994. Transgenic potato plants with strongly decreased expression of pyrophosphate: fructose-6-phosphate phosphotransferase show no visible phenotype and only minor changes in metabolic fluxes in their tubers. Planta 192, 16-30.

Halford, N.G., Purcell, P.C., Hardie, D.G., 1999. Is hexokinase really a sugar sensor in plants? Trends Plant Sci. 4, 117-120.

Harrington, G.N., Bush, D.R., 2003. The bifunctional role of hexokinase in metabolism and glucose signaling. Plant Cell 15, 2493-2496.

Häusler, R.E., Holtum, J.A.M., Latzko, E., 1989. Cytosolic phosphofructokinase from spinach leaves. Plant Physiol. 90, 1498-1505.

Heazlewood, J.L., Verboom, R.E., Tonti-Filippini, J., Small, I., Millar, A.H., 2007. SUBA: the *Arabidopsis* subcellular database. Nucleic Acids Res. 35, D213-D218.

Heinrich, R., Rapoport, T.A., 1974. A linear steady-state treatment of enzymatic chains. General properties, control and effector strength. Eur. J. Biochem. 42, 89-95.

Hill, S.A., ap Rees, T., 1994. Fluxes of carbohydrate metabolism in ripening bananas. Planta 192, 52-60.

Hofmeyr, J.H., Cornish-Bowden, A., 1991. Quantitative assessment of regulation in metabolic systems. Eur. J. Biochem. 200, 223-236.

Huang, S., Greenway, H., Colmer, T.D., Millar, A.H., 2005. Protein synthesis by rice coleoptiles during prolonged anoxia: Implications for glycolysis, growth and energy utilization. Ann. Bot. 96, 703-715.

Huppe H.C., Turpin, D.H., 1994. Integration of carbon and nitrogen metabolism in plant and algal cells. Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 577-607.

Igamberdiev, A.U., Baron, K., Manac'h-Little, N., Stoimenova, M., Hill, R.D., 2005. The haemoglobin/nitric oxide cycle: Involvement in flooding stress and effects on hormone signalling. Ann. Bot. 96, 557-564.

Iynedjian, P.B., 1998. Glycolysis, turbo design and the endocrine pancreatic β cell. Trends Biochem. Sci. 23, 467-468.

Jang, J.C., Leon, P., Zhou, L., Sheen, J., 1997. Hexokinase as a sugar sensor in higher plants. Plant Cell 9, 5-19.

Junker, B.H., Wuttke, R., Nunes-Nesi, A., Steinhauser, D., Schauer, N., Bussis, D., Willmitzer, L., Fernie, A.R., 2006. Enhancing vacuolar sucrose cleavage within the developing potato tuber has only minor effects on metabolism. Plant Cell Physiol. 47, 277-289.

Kacser, H., Burns, J.A., 1973. The control of flux. Symp. Soc. Exp. Biol. 27, 65-104.

Kacser, H., Burns, J.A., Fell, D.A., 1995. The control of flux: 21 years on. Biochem. Soc. Trans. 23, 341-366.

Kandel-Kfir, M., Damari-Weissler, H., German, M.A., Gidoni, D., Mett, A., Belausov, E., Petreikov, M., Adir, N., Granot, D., 2006. Two newly identified membrane-associated and plastidic tomato HXKs: Characteristics, predicted structure and intracellular localization. Planta 224, 1341-1352.

Kashiwaya, Y., Sato, K., Tsuchiya, N., Thomas, S., Fell, D.A., Veech, R.L., Passonneau, J.V., 1994. Control of glucose utilization in working perfused rat heart. J. Biol. Chem. 269, 25502-25514.

Kelley, P.M., Freeling, M., 1984. Anaerobic expression of maize glucose phosphate isomerase I. J. Biol. Chem. 259, 673-677.

Kim, J.W., Dang, C.V., 2005. Multifaceted roles of glycolytic enzymes. Trends Biochem. Sci. 30, 142-150.

Kim, M., Lim, J.H., Ahn, C.S., Park, K., Kim, G.T., Kim, W.T., Pai, H.S., 2006. Mitochondria-associated hexokinases play a role in the control of programmed cell death in *Nicotiana benthamiana*. Plant Cell 18, 2341-2355.

Klok, E.J., Wilson, I.W., Wilson, D., Chapman, S.C., Ewing, R.M., Somerville, S.C., Peacock, W.J., Dolferus, R., Dennis, E.S., 2002. Expression profile analysis of the low-oxygen response in *Arabidopsis* root cultures. Plant Cell 14, 2481-2494.

Koch, K.E., 1996. Carbohydrate-modulated gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 509-540.

Koebmann, B.J., Westerhoff, H.V., Snoep, J.L., Nilsson, D., Jensen, P.R., 2002. The glycolytic flux in *Escherichia coli* is controlled by the demand for ATP. J. Bacteriol. 184, 3909-3916.

Komatsu, S., Konishi, H., Hashimoto, M., 2006. The proteomics of plant cell membranes. J. Exp. Bot. 58, 103-112.

Kresge, N., Simoni, R.D., Hill, R.L., 2005. Otto Fritz Meyerhof and the elucidation of the glycolytic pathway. J. Biol. Chem. 280, e3 (124-126).

Kruger, N.J., von Schaewen, A., 2003. The oxidative pentose phosphate pathway: structure and organisation. Curr. Opin. Plant Biol. 6, 236-246.

Lalonde, S., Ehrhardt, D.W., Frömmer, W.B., 2005. Shining light on signaling and metabolic networks by genetically encoded biosensors. Curr. Opin. Plant Biol. 8, 574-581.

Lange, A.J., 2007. For the ZDF rat, "breaking up is hard to do": Dissociation of the GK:GKRP complex. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292, R1379-R1380.

Larsson, C., Pahlman, I.L., Gustafsson, L., 2000. The importance of ATP as a regulator of glycolytic flux in *Saccharomyces cerevisiae*. Yeast 16, 797-809.

Law R.D., Plaxton, W.C., 1995. Purification and characterization of a novel phospho*enol*pyruvate carboxylase from banana fruit. Biochem. J. 307, 807-816.

Lee, D., Ezhkova, E., Li, B., Pattenden, S.G., Tansey, W.P., Workman, J.L., 2005. The proteasome regulatory particle alters the SAGA coactivator to enhance its interactions with transcriptional activators. Cell 123, 423-436.

Lejay, L., Gansel, X., Cerezo, M., Tillard, P., Muller, C., Krapp, A,, von Wiren, N., Daniel-Vedele, F., Gojon, A., 2003. Regulation of root ion transporters by photosynthesis: Functional importance and relation with hexokinase. Plant Cell 15, 2218-2232.

Lin, J., Wang, Y., Wang, G., 2006. Salt stress-induced programmed cell death in tobacco protoplasts is mediated by reactive oxygen species and mitochondrial permeability transition pore status. J. Plant Physiol. 163, 731-739.

Liu, F., Vantoai, T., Moy, L.P., Bock, G., Linford, L.D., Quackenbush, J., 2005. Global transcription profiling reveals comprehensive insights into hypoxic response in *Arabidopsis*. Plant Physiol. 137, 1115-1129.

Liu, L.M., Li, Y., Du, G.C., Chen, J., 2006. Increasing glycolytic flux in *Torulopsis glabrata* by redirecting ATP production from oxidative phosphorylation to substrate-level phosphorylation. J. Appl. Microbiol. 100, 1043-1053.

Loreti, E., Poggi, A., Novi, G., Alpi, A., Perata, P., 2005. A genome-wide analysis of the effects of sucrose on gene expression in *Arabidopsis* seedlings under anoxia. Plant Physiol. 137, 1130-1138.

Marmagne, A., Rouet, M.-A., Ferro, M., Rolland, N., Alcon, C., Joyard, J., Garin, J., Barbier-Brygoo, H., Ephritikhine, G., 2004. Identification of new intrinsic proteins in *Arabidopsis* plasma membrane proteome. Mol. Cell Proteomics 3, 675-691.

McCue, K.F., Hanson, A.D., 1990. Drought and salt tolerance: Towards understanding and application. Trends Biotechnol. 8, 358-362.

Meléndez-Hevia, E., Mateo, F., Torres, N.V., 1992. Control analysis of rat liver glycolysis under different glucose concentrations. The substrate approach and the role of glucokinase. Mol. Cell Biochem. 115, 1-9.

Menu, T., Saglio, P., Granot, D., Dai, N., Raymond, P., Ricard, B., 2004. High hexokinase activity in tomato fruit perturbs carbon and energy metabolism and reduces fruit and seed size. Plant Cell Environ. 27, 89-98.

Mertens, E., Larondelle, Y., Hers, H.G., 1990. Induction of pyrophosphate:fructose 6-phosphate 1-phosphotransferase by anoxia in rice seedlings. Plant Physiol. 93, 584-587.

Meyerhof, O., 1927. Über die enzymatische Milchsäurebildung im Muskelextrakt. III. Mitteilung : Die Milchäurebildung aus den gärfähigen Hexosen. Biochem. Z. 183, 176-215.

Miernyk, J.A., Dennis, D.T., 1983. Mitochondrial, plastid, and cytosolic isozymes of hexokinase from developing endosperm of *Ricinus communis*. Arch. Biochem. Biophys. 226, 458-468.

Moons, A., Valcke, R., Van Montagu, M., 1998. Low-oxygen stress and water deficit induce cytosolic pyruvate orthophosphate dikinase (PPDK) expression in roots of rice, a C_3 plant. Plant J. 15, 89-98.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Liu, Y.X., Hwang, I., Jones, T., Sheen, J., 2003. Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science 300, 332-336.

Neuhaus, H.E., Emes, M.J., 2000. Nonphotosynthetic metabolism in plastids. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 111-140.

Nguyen-Quoc, B., Foyer, C.H., 2001. A role for 'futile cycles' involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. J. Exp. Bot. 52, 881-889.

Olsson, T., Thelander, M., Ronne, H., 2003. A novel type of chloroplast stromal hexokinase is the major glucose-phosphorylating enzyme in the moss *Physcomitrella patens*. J. Biol. Chem. 278, 44439-44447.

Pastorino, J.G., Hoek, J.B., Shulga, N., 2005. Activation of glycogen synthase kinase 3β disrupts the binding of hexokinase II to mitochondria by phosphorylating voltage-dependent anion channel and potentiates chemotherapy-induced cytotoxicity. Cancer Res. 65, 10545-10554.

Paul, M., 2007. Trehalose 6-phosphate. Curr. Opin. Plant Biol. 10, 303-309.

Plaxton, W.C., 1996. The organization and regulation of plant glycolysis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 185-214.

Podestá, F.E., Plaxton, W.C., 1994. Regulation of carbon metabolism in germinating *Ricinus communis* cotyledons. II. Properties of phospho*enol*pyruvate carboxylase and cytosolic pyruvate kinase associated with the regulation of glycolysis and nitrogen assimilation. Planta 194, 381-387.

Poolman, M.G., Fell, D.A., Thomas, S., 2000. Modelling photosynthesis and its control. J. Exp. Bot. 51, 319-328.

Portais, J.C., Delort, A.M., 2002. Carbohydrate cycling in micro-organisms: What can ^{13}C-NMR tell us? FEMS Microbiol. Rev. 26, 375-402.

Puigjaner, J., Rais, B., Burgos, M., Comin, B., Ovadi, J., Cascante, M., 1997. Comparison of control analysis data using different approaches: Modelling and experiments with muscle extract. FEBS Lett. 418, 47-52.

Quick, W.P., Schaffer, A.A., 1996. Sucrose metabolism in sources and sinks. In: Zamski, E., Schaffer, A.A. (Eds.), *Photoassimilate Distribution in Plants and Crops. Source-Sink Relationships*. M. Dekker Inc., New York, NY, USA, pp. 115-156.

Rapoport, T.A., Heinrich, R., Jacobasch, G., Rapoport, S.M., 1974. A linear steady-state treatment of enzymatic chains. A mathematical model of glycolysis of human erythrocytes. Eur. J. Biochem. 42, 107-120.

Renz, A., Merlo, L., Stitt, M., 1993. Partial purification from potato tubers of three fructokinases and three hexokinases which show differing organ and developmental specificity. Planta 190, 156-165.

Renz, A., Stitt, M., 1993. Substrate specificity and product inhibition of different forms of fructokinases and hexokinases in developing potato tubers. Planta 190, 166-175.

Rezende, G.L., Logullo, C., Meyer, L., Machado, L.B., Oliveira-Carvalho, A.L., Zingali, R.B., Cifuentes, D., Galina, A., 2006. Partial purification of tightly bound mitochondrial hexokinase from maize (*Zea mays* L.) root membranes. Braz. J. Med. Biol. Res. 39, 1159-1169.

Ricard, B., Couée, I., Raymond, P., Saglio, P.H., Saint-Ges, V., Pradet, A., 1994. Plant metabolism under hypoxia and anoxia. Plant Physiol. Biochem. 32, 1-10.

Ricard, B., Rivoal, J., Spiteri, A., Pradet, A., 1991. Anaerobic stress induces the transcription and translation of sucrose synthase in rice. Plant Physiol. 95, 669-674.

Rivoal, J., Dorion, S., Claeyssen, E., Zhang, H., 2004. Organization of the plant cytosolic glycolytic pathway and its regulation by abiotic stresses in nonphotosynthetic tissues. In: Pandalai, S.G. (Ed.), *Recent Research Developments in Environmental Biology*, pp. 77-100.

Rivoal, J., Hanson, A.D., 1993. Evidence for a large and sustained glycolytic flux to lactate in anoxic roots of some members of the Halophytic genus *Limonium*. Plant Physiol. 101, 553-560.

Rivoal, J., Ricard, B., Pradet, A., 1989. Glycolytic and fermentative enzyme induction during anaerobiosis in rice seedlings. Plant Physiol. Biochem. 27, 43-52.

Roessner-Tunali, U., Hegemann, B., Lytovchenko, A., Carrari, F., Bruedigam, C., Granot, D., Fernie, A.R., 2003. Metabolic profiling of transgenic tomato plants overexpressing hexokinase reveals that the influence of hexose phosphorylation diminishes during fruit development. Plant Physiol. 133, 84-99.

Rolland, F., Baena-Gonzalez, E., Sheen, J., 2006. Sugar sensing and signaling in plants: Conserved and novel mechanisms. Annu. Rev. Plant Biol. 57, 675-709.

Rolland, F., Moore, B., Sheen, J., 2002. Sugar sensing and signaling in plants. Plant Cell 14 (Suppl.), S185-S205.

Rolland, F., Winderickx, J., Thevelein, J.M., 2001. Glucose-sensing mechanisms in eukaryotic cells. Trends Biochem. Sci. 26, 310-317.

Rontein, D., Dieuaide-Noubhani, M., Dufourc, E.J., Raymond, P., Rolin, D., 2002. The metabolic architecture of plant cells. Stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. J. Biol. Chem. 277, 43948-43960.

Roscher, A., Emsley, L., Raymond, P., Roby, C., 1998. Unidirectional steady state rates of central metabolism enzymes measured simultaneously in a living plant tissue. J. Biol. Chem. 273, 25053-25061.

Russell, D.A., Sachs, M.M., 1989. Differential expression and sequence-analysis of the maize glyceraldehyde-3-phosphate dehydrogenase gene family. Plant Cell 1, 793-803.

Ruuska, S.A., Girke, T., Benning, C., Ohlrogge, J.B., 2002. Contrapuntal networks of gene expression during *Arabidopsis* seed filling. Plant Cell 14, 1191-1206.

Salon, C., Raymond, P., Pradet, A., 1988. Quantification of carbon fluxes through the tricarboxylic acid cycle in early germinating lettuce embryos. J. Biol. Chem. 263, 12278-12287.

Saltman, P., 1953. Hexokinase in higher plants. J. Biol. Chem. 200, 145-154.

Santoni, V., Rouquié, D., Doumas, P., Mansion, M., Boutry, M., Degand, H., Dupree, P., Packmann, L., Sherrier, J., Prime, T., Bauw, G., Posada, E., Rouzé, P., Dehais, P., Sahnoun, I., Barlier, I., Rossignol, M., 1998. Use of a proteome strategy for tagging proteins present at the plasma membrane. Plant J. 16, 633-641.

Schnarrenberger, C., 1990. Characterization and compartmentation, in green leaves, of hexokinases with different specificities for glucose, fructose, and mannose and for nucleoside triphosphates. Planta 181, 249-255.

Schuller, K.A., Turpin, D.H., Plaxton, W.C., 1990. Metabolite regulation of partially purified soybean nodule PEP carboxylase. Plant Physiol. 94, 1429-1435.

Schwender, J., Ohlrogge, J.B., 2002. Probing *in vivo* metabolism by stable isotope labeling of storage lipids and proteins in developing *Brassica napus* embryos. Plant Physiol. 130, 347-361.

Schwender, J., Ohlrogge, J.B., Shachar-Hill, Y., 2003. A flux model of glycolysis and the oxidative pentosephosphate pathway in developing *Brassica napus* embryos. J. Biol. Chem. 278, 29442-29453.

Shin, J.S., Torres, T.P., Catlin, R.L., Donahue, E.P., Shiota, M., 2007. A defect in glucose-induced dissociation of glucokinase from the regulatory protein in Zucker diabetic fatty rats in the early stage of diabetes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292, R1381-R1390.

Siedow, J.N., Day, D.A., 2000. Respiration and photorespiration. In: Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.), *Biochemistry and Molecular Biology of Plants*. Am. Soc. Plant Physiol., Rockville, MA, USA, pp. 676-729.

Small, J.R., Kacser, H., 1993. Responses of metabolic systems to large changes in enzyme activities and effectors. 2. The linear treatment of branched pathways and metabolite concentrations. Assessment of the general non-linear case. Eur. J. Biochem. 213, 625-640.

Smirnoff, N., 1995. Metabolic flexibility in relation to the environment. In: Smirnoff, N. (Ed), *Environment and Plant Metabolism. Flexibility and Acclimation.* Bios Scientific Publishers, Oxford, England, pp. 1-16.

Smith, A.M., Zeeman, S.C., Smith, S.M., 2005. Starch degradation. Annu. Rev. Plant Biol. 56, 73-98.

Spielbauer, G., Margl, L., Hannah, L.C., Romisch, W., Ettenhuber, C., Bacher, A., Gierl, A., Eisenreich, W., Genschel, U., 2006. Robustness of central carbohydrate metabolism in developing maize kernels. Phytochem. 67, 1460-1475.

Stitt, M., Sonnewald, U., 1995. Regulation of metabolism in transgenic plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 341-368.

Sweet, I.R., Matschinsky, F.M., 1995. Mathematical model of β-cell glucose metabolism and insulin release. I. Glucokinase as glucosensor hypothesis. Am. J. Physiol. 268, E775-E788.

Sweetlove, L.J., Müller-Röber, B., Willmitzer, L., Hill, S.A., 1999. The contribution of adenosine 5'-diphosphoglucose pyrophosphorylase to the control of starch synthesis in potato tubers. Planta 209, 330-337.

Sweetlove, L.J., Tomlinson, K.L., Hill, S.A., 2002. The effect of exogenous sugars on the control of flux by adenosine 5'-diphosphoglucose pyrophosphorylase in potato tuber discs. Planta 214, 741-750.

Tanaka, N., Fujita, M., Handa, H., Murayama, S., Uemura, M., Kawamura, Y., Mitsui, T., Mikami, S., Tozawa, Y., Yoshinaga, T., Komatsu, S., 2004. Proteomics of the rice cell: Systematic identification of the protein populations in subcellular compartments. Mol. Gen. Genomics 271, 566-576.

Tanner, G.J., Copeland, L., Turner, J.F., 1983. Subcellular localization of hexose kinases in pea stems: Mitochondrial hexokinase. Plant Physiol. 72, 659-663.

Tetlow, I.J., Rawsthorne, S., Raines, C., Emes, M.J., 2005. Plastid metabolic pathways. In: Moller, S.G. (Ed.), *Plastids*. Blackwell Publishing Ltd, England, pp. 60-125.

Teusink, B., Walsh, M.C., van Dam, K., Westerhoff, H.V., 1998. The danger of metabolic pathways with turbo design. Trends Biochem. Sci. 23, 162-169.

Theodorou, M.E., Plaxton, W.C., 1993. Metabolic adaptations of plant respiration to nutritional phosphate deprivation. Plant Physiol. 101, 339-344.

Thevelein, J.M., Hohmann, S., 1995. Trehalose synthase: Guard to the gate of glycolysis in yeast? Trends Biochem. Sci. 20, 3-10.

Thomas, S., Fell, D.A., 1998. A control analysis exploration of the role of ATP utilisation in glycolytic-flux control and glycolytic-metabolite-concentration regulation. Eur. J. Biochem. 258, 956-967.

Thomas, S., Mooney, P.J., Burrell, M.M., Fell, D.A., 1997. Metabolic Control Analysis of glycolysis in tuber tissue of potato (*Solanum tuberosum*): Explanation for the low control coefficient of phosphofructokinase over respiratory flux. Biochem. J. 322, 119-127.

Trethewey, R.N., Riesmeier, J.W., Willmitzer, L., Stitt, M., Geigenberger, P., 1999. Tuber-specific expression of a yeast invertase and a bacterial glucokinase in potato leads to an activation of sucrose phosphate synthase and the creation of a sucrose futile cycle. Planta 208, 227-238.

Turner, J.F., Chensee, Q.J., Harrison, D.D., 1977. Glucokinase of pea seeds. Biochim. Biophys. Acta 480, 367-375.

Urbanczyk-Wochniak, E., Leisse, A., Roessner-Tunali, U., Lytovchenko, A., Reismeier, J., Willmitzer, L., Fernie, A.R., 2003. Expression of a bacterial xylose isomerase in potato tubers results in an altered hexose composition and a consequent induction of metabolism. Plant Cell Physiol. 44, 1359-1367.

Vartapetian, B.B., Andreeva, I.N., Generozova, I.P., Polyakova, L.I., Maslova, I.P., Dolgikh, Y.I., Stepanova, A.Y., 2003. Functional electron microscopy in studies of plant response and adaptation to anaerobic stress. Ann. Bot.(Lond) 91, 155-172.

Vella, J., Copeland, L., 1993. Phosphofructokinase from the host fraction of soybean nodules. J. Plant Physiol. 141, 398-404.

Veramendi, J., Fernie, A.R., Leisse, A., Willmitzer, L., Trethewey, R.N., 2002. Potato hexokinase 2 complements transgenic *Arabidopsis* plants deficient in hexokinase 1 but does not play a key role in tuber carbohydrate metabolism. Plant Mol. Biol. 49, 491-501.

Veramendi, J., Roessner, U., Renz, A., Willmitzer, L., Trethewey, R.N., 1999. Antisense repression of hexokinase 1 leads to an overaccumulation of starch in leaves of transgenic potato plants but not to significant changes in tuber carbohydrate metabolism. Plant Physiol. 121, 123-134.

Wang, H., Iynedjian, P.B., 1997a. Modulation of glucose responsiveness of insulinoma β-cells by graded overexpression of a glucokinase. Proc. Natl. Acad. Sci. USA 94, 4372-4377.

Wang, H., Iynedjian, P.B., 1997b. Acute glucose intolerance in insulinoma cells with unbalanced overexpression of glucokinase. J. Biol. Chem. 272, 25731-25736.

Wang, R., Okamoto, M., Xing, X., Crawford, N.M., 2003. Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol. 132, 556-567.

Weber, A.P.M., Schwacke, R., Flügge, U.I., 2005. Solute transporters of the plastid envelope membrane. Annu. Rev. Plant Biol. 56, 133-164.

Wiese, A., Groner, F., Sonnewald, U., Deppner, H., Lerchl, J., Hebbeker, U., Flügge, U., Weber, A., 1999. Spinach hexokinase I is located in the outer envelope membrane of plastids. FEBS Lett. 461, 13-18.

Wilson, J.E., 2003. Isozymes of mammalian hexokinase: Structure, subcellular localization and metabolic function. J. Exp. Biol. 206, 2049-2057.

Xiao, W., Sheen, J., Jang, J.C., 2000. The role of hexokinase in plant sugar signal transduction and growth and development. Plant Mol. Biol. 44, 451-461.

Yan, S., Tang, Z., Su, W., Sun, W., 2005. Proteomic analysis of salt stress-responsive proteins in rice roots. Proteomics 5, 235-244.

Yanagisawa, S., Yoo, S.D., Sheen, J., 2003. Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature 425, 521-525.

Yao, N., Eisfelder, B.J., Marvin, J., Greenberg, J.T., 2004. The mitochondrion - An organelle commonly involved in programmed cell death in *Arabidopsis thaliana*. Plant J. 40, 596-610.

Zeng, Y., Wu, Y., Avigne, W.T., Koch, K.E., 1998. Differential regulation of sugar-sensitive sucrose synthases by hypoxia and anoxia indicate complementary transcriptional and posttranscriptional responses. Plant Physiol. 116, 1573-1583.

Annexe 1.

Codes EC des enzymes citées

Enzyme	Abréviation	Code EC[a]
3-phospho-D-glycérate kinase	3-PGA kinase	EC 2.7.2.3
3-phospho-D-glycérate mutase	3-PGA mutase	EC 5.4.2.1
α-amylase		EC 3.2.1.1
β-amylase		EC 3.2.1.2
α-galactosidase		EC 3.2.1.22
α-glucosidase		EC 3.2.1.20
ADP-D-glucose pyrophosphorylase	AGPase	EC 2.7.7.27
aldolase	ALD	EC 4.1.2.13
2-désoxy-D-glucose-6-phosphatase		EC 3.1.3.68
énolase		EC 4.2.1.11
enzyme malique NAD-dépendante, mitochondriale		
	NAD-ME	EC 1.1.1.39
enzyme malique NADP-dépendante, cytosolique ou plastidique		
	NADP-ME	EC 1.1.1.40
fructokinase	FK	EC 2.7.1.4
D-fructose-1,6-bisphosphatase	FBPase	EC 3.1.3.11
G3P déshydrogénase NAD-dépendante, phosphorylante, cytosolique		
	NAD-GAPDH(P)	EC 1.2.1.12
G3P déshydrogénase NADP-dépendante, phosphorylante, plastidique		
	NADP-GAPDH(P)	EC 1.2.1.13
G3P déshydrogénase NAD(P)-dépendante, phosphorylante, cytosolique		
	NAD(P)-GAPDH(P)	EC 1.2.1.59
G3P déshydrogénase NADP-dépendante, non phosphorylante, cytosolique		
	NADP-GAPDH(NP)	EC 1.2.1.9

D-glucose-1-phosphatase		EC 3.1.3.10
D-glucose-6-phosphatase		EC 3.1.3.9
D-glucose-6-phosphate déshydrogénase	G6PDH	EC 1.1.1.49
GDP-D-mannose pyrophosphorylase	GMPase	EC 2.7.7.13
galactokinase	GalK	EC 2.7.1.6
glucokinase	GK	EC 2.7.1.2
Glu synthase	GOGAT	EC 1.4.1.13
Gln synthétase	GS	EC 6.3.1.2
hexokinase	HK	EC 2.7.1.1
invertase	INV	EC 3.2.1.26
lactate déshydrogénase	LDH	EC 1.1.1.28
malate déshydrogénase	MDH	EC 1.1.1.37
mannitol déshydrogenase	MTD	EC 1.1.1.255
mannokinase	MK	EC 2.7.1.7
nitrate réductase	NR	EC 1.7.1.1
nucléoside-5'-diphosphate kinase	NDPK	EC 2.7.4.6
phosphatase acide		EC 3.1.3.2
phosphatase alcaline (bactérienne)		EC 3.1.3.1
phospho*énol*pyruvate carboxylase	PEPC	EC 4.1.1.31
phospho*énol*pyruvate phosphatase	PEPase	EC 3.1.3.60
phosphofructokinase ATP-dépendante	PFK	EC 2.7.1.11
phosphoglucose isomérase	PGI	EC 5.3.1.9
phosphoglucomutase	PGM	EC 5.4.2.2
phosphomannose isomérase	PMI	EC 5.3.1.8
phosphomannomutase	PMM	EC 5.4.2.8
phosphorylase		EC 2.4.1.1
pyrophosphate-F6P 1-phosphotransférase	PFP	EC 2.7.1.90
pyruvate kinase	PK	EC 2.7.1.40
pyruvate, phosphate dikinase	PPDK	EC 2.7.9.1
D-saccharose-phosphate phosphatase		EC 3.1.3.24
D-saccharose phosphorylase		EC 2.4.1.7

D-saccharose-phosphate synthase	SPS	EC 2.4.1.14
D-saccharose synthase	SuSy	EC 2.4.1.13
Suc-Suc fructosyltransférase	SST	EC 2.4.1.99
transglucosidase	TGD	EC 2.4.1.25
triose-phosphate isomérase	TPI	EC 5.3.1.1
UDP-D-glucose pyrophosphorylase	UGPase	EC 2.7.7.9
D-xylose isomérase		EC 5.3.1.5

[a] Les codes EC ont été vérifiés sur le site Expert Protein Analysis System (ExPASy) (http://ca.expasy.org/, Gasteiger *et al.*, 2003).